



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁷ : A61K 35/78, A61P 31/12	A2	(11) International Publication Number: WO 00/61161 (43) International Publication Date: 19 October 2000 (19.10.00)
(21) International Application Number: PCT/IN00/00046 (22) International Filing Date: 10 April 2000 (10.04.00) (30) Priority Data: 405/MAS/99 12 April 1999 (12.04.99) IN (71) Applicant (for all designated States except US): UNIVERSITY OF MADRAS [IN/IN]; Kamarajar Salai, Chepauk, Chennai 600 005 (IN). (72) Inventor; and (75) Inventor/Applicant (for US only): THYAGARAJAN, Sadras, Panchatcharam [IN/IN]; Old No.3, New No. 5, First Street, Nehru Nagar, Adyar, Chennai 600 020 (IN).		(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>Without international search report and to be republished upon receipt of that report.</i>
(54) Title: A PHARMACEUTICAL FORMULATION USEFUL FOR THE TREATMENT OF HEPATITIS B, HEPATITIS C AND OTHER VIRAL INFECTIONS OF THE LIVER AND A PROCESS FOR ITS PREPARATION (57) Abstract <p>The invention disclosed in this application relates to a pharmaceutical formulation prepared from the biotyped variety of the medicinal plant, <i>Phyllanthus amarus</i> which is useful for the treatment of Hepatitis B (both acute and chronic), Hepatitis C (both acute and chronic) and other related viral infections of the liver with antihepatotoxic and liver cell regenerating potentials and immunomodulating properties. The invention confirms, that when parts of the biotyped variety of the medicinal plant, <i>Phyllanthus amarus</i> are extracted separately with a polar solvent alone, polar solvent and water in specific ratios and water alone and when such extracts are mixed together, the resultant formulation has all the essential antiviral and biological properties, while the individual polar or aqueous extracts alone does not possess one or more of these properties. The present invention also relates to a process for the preparation of the above said new pharmaceutical formulation with biological and chemical standardisation protocols.</p>		

BEST AVAILABLE COPY

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

A pharmaceutical formulation useful for the treatment of Hepatitis B, Hepatitis C and other viral infections of the liver and a process for its preparation

Technical Field

This invention relates to a pharmaceutical formulation useful for the treatment of Hepatitis B and Hepatitis C and other viral infections of liver. This invention particularly relates to a pharmaceutical formulation useful for the treatment of acute and chronic Hepatitis B & C virus infections prepared from the Indian biotyped medicinal plant, *Phyllanthus amarus*. This invention also relates to a process for the preparation of the pharmaceutical formulation useful for the treatment of acute and chronic Hepatitis B and Hepatitis C and other viral infections of the liver from the medicinal plant *Phyllanthus amarus*.

It is needless to stress the need for a successful drug that would keep the liver functioning at its optimum or the one that would be selectively active against the currently known etiological agents of acute and chronic viral diseases of the liver. This is important because the disease of the liver throw the entire human body out of gear. The exciting alphabet of viral Hepatitis includes a wide range of totally unrelated often highly unusual pathogenic human viruses like Hepatitis A virus (HAV), Hepatitis B virus (HBV), Hepatitis C virus (HCV), Hepatitis D virus (HDV), Hepatitis E virus (HEV) etc. Of the viruses it has been clearly established that HBV, HCV and HDV are the ones that are associated with the development of chronic persistent/active hepatitis, cirrhosis of the liver and even hepatocellular carcinoma besides being associated with fulminant hepatitis and sub acute hepatic failure.

Background Art

ACUTE AND CHRONIC HEPATITIS-B

The natural disease course in HBV is being summarized to understand the need for the effective management and treatment of Hepatitis B in a country. For normal adults with low viral production and an early immune response, the disease course is self limiting and usually asymptomatic (60-80% of all HBV infections). Individuals who replicate the virus in larger quantities, with a relatively late immune response have a self-limiting symptomatic acute

hepatitis. Irrespective of whether initially symptomatic or asymptomatic, the infection becomes chronic in 5-10% of the individuals, 20-30% of them developing clinical sequelae such as chronic hepatitis, cirrhosis or hepatoma within years or decades. In neonates, however, the immune defence is still lacking (induction of tolerance), so that infected individuals do not develop acute hepatitis, but more of them become chronic carriers (80-90%). Such carriers also progress frequently to chronic clinical sequelae faster. Between these two extremes are immunocompromised individuals, such as intravenous drug users, haemodialysis patients or transplant recipients, who are more likely to become chronic carriers than are healthy adults (10-60%). (WHO Tech. Report. Series 1987;754:18)

Based on substantial body of data, HBV has been proved as a major pathogen producing chronic liver diseases. It has also been proved that there are over 400 million healthy carriers of HBV all over the world and one tenth of these carriers (40 millions) being in India alone. These carriers besides acting as human reservoirs of HBV infection also act as primary source of spread of HBV infection to the community and was also shown to have 200 times increased risk of developing chronic liver diseases and/or hepatocellular carcinoma.

With the above documented international HBV scenario, the HBV epidemiology in India is to be considered as alarming since there are definite data on prevalence pattern of HBV in asymptomatic population (4%) high risk groups (13%), significant involvement of HBV in Indian acute and sub-acute liver failure cases (42 & 45%). 70% of the chronic hepatitis cases, 40-80% of cirrhosis cases and over 60% of primary liver cancer cases.

Although effective vaccines have been developed against HBV and successfully adopted, the need for effective treatment of acute and chronic Hepatitis B has become universal public health emergency since vaccines as on date are neither capable of inducing immunity in a carrier nor able to eliminate HBV carrier status. Research conducted from the mid 70s have delineated several agents to have treatment potential in chronic HBV infections which has been illustrated in the Table 1 given below.

Table 1Agents that have been studied in the treatment of HBV infection

(Lau et al., Gut. Suppl. 1991;S47-S62)

Anti-virals	Immunosuppressive
Interferons	Corticosteroids
Alpha interferon	
Beta interferon	
Gamma interferon	
Tumour necrosis factor	
Adenine arabinoside (Ara-A)	Immunostimulators
Acyclovir, deoxyacydovir	BCG vaccination
Zidovudine	Levamisole
Suramin	Interleukin-2
Ribavirin	Interferon-gamma
Phosphonoformate	Thymosin
Quinacrine	Tumor necrosis factor
(+) -cyanidanol-3	
Lamuvudine	
Phyllanthus amarus	

However, except the interferons, Lamuvudine and the latest entry Phyllanthus amarus, the others seem to be far from successful. The limited success rate, prohibitive cost, profound side effects and the non-accessibility of interferons and Lamuvudine in developing and underdeveloped countries have necessitated further search for newer antihepatitis B agents.

ACUTE AND CHRONIC HEPATITIS-C:**1. Acute HCV Infection**

Persons with acute HCV infection typically are either asymptomatic or have a mild clinical illness ; 60%-70% have no discernible symptoms ; 20%-30% might have jaundice ; and 10%-20% might have nonspecific symptoms (e.g. anorexia, malaise or abdominal pain). Clinical illness in patients with acute hepatitis C who seek medical care is similar to that of other types

of viral hepatitis, and serologic testing is necessary to determine the etiology of hepatitis in an individual patient. In $\geq 20\%$ of these patients, onset of symptoms might precede anti-HCV seroconversion. Average time period from exposure to symptom onset is 6-7 weeks, whereas average time period from exposure to seroconversion is 8-9 weeks. Anti-HCV can be detected in 80% of patients within 15 weeks after exposure, in $\geq 90\%$ within 5 months after exposure, and in $\geq 97\%$ by 6 months after exposure. Rarely, seroconversion might be delayed until 9 months after exposure.

The course of acute hepatitis C is variable, although elevations in serum ALT levels often in a fluctuating pattern, are its most characteristic feature. Normalization of ALT levels might occur and suggests full recovery, but this is frequently followed by ALT elevations that indicate progression to chronic disease. Fulminant hepatic failure following acute hepatitis C is rare. However, in developing country especially India, HCV in FHF was reported significantly.

2. Chronic HCV Infection

After acute infection, 15%-25% of persons appear to resolve their infection without sequelae as defined by sustained absence of HCV RNA in serum and normalization of ALT levels. Chronic HCV infection develops in most persons (75%-85%), with persistent or fluctuating ALT elevations indicating active liver disease developing in 60%-70% of chronically infected persons. In the remaining 30%-40% of chronically infected persons, ALT levels are normal. No clinical or epidemiologic features among patients with acute infection have been found to be predictive of either persistent infection or chronic liver disease. Moreover, various ALT patterns have been observed in these patients during follow-up, and patients might have prolonged periods (≥ 12 months) of normal ALT activity even though they have histologically confirmed chronic hepatitis. Thus a single ALT determination cannot be used to exclude ongoing hepatic injury, and long term follow-up of patients with HCV infection is required to determine their clinical outcome or prognosis.

The course of chronic liver disease is usually insidious, progressing at a slow rate without symptoms or physical signs in the majority of patients during the first two or more decades after infection. Frequently, chronic hepatitis C is not recognised until asymptomatic persons are identified as HCV positive during blood donor screening or elevated ALT levels are

detected during routine physical examinations. Most studies have reported that cirrhosis develops in 10%-20% of persons with chronic hepatitis C over a period of 20-30 years, and

HCC in 1%-5%, with striking geographic variations in rates of this disease. This difference is more discernable also in the SEA region countries.

Although factors predicting severity of liver disease have not been well defined, recent data indicate that increased alcohol intake, being aged >40 years at infection, and being male are associated with more severe liver disease. In particular, among persons with alcoholic liver disease and HCV infection, liver disease progresses more rapidly ; among those with cirrhosis, a higher risk for development of HCC exists. Furthermore, even intake of moderate amounts (>10 g/day) of alcohol in patients with chronic hepatitis C might enhance disease progression. More severe liver injury observed in persons with alcoholic liver disease and HCV infection possibly is attributable to alcohol induced enhancement of viral replication or increased susceptibility of cells to viral injury.

The current treatment guidelines of HCV infection are presented below :

- Current effective therapy for HCV infection is IFN based, with or without other therapeutic agents such as ribavirin. Ribavirin monotherapy is not recommended.
- For the interim, patients with acute hepatitis should receive IFN- α , 3-6 million units (or 9-15 μ g) thrice weekly for atleast 6 months until more effective regimens emerge.
- The standard treatment for previously untreated (naïve) patients with chronic hepatitis C is IFN- α , 3-6 million units (or 9-15 μ g) thrice weekly for 12 months. However, recent data indicate that a regimen of IFN- α and ribavirin for 6 months or IFN- α monotherapy using different schedules and/or higher doses may significantly improve sustained response rates and become preferred options for treatment in the future.
- Adverse side-effects to IFN and ribavirin are tolerable, but a fatal outcome (suicide, liver failure, sepsis) has been observed primarily in patients with cirrhosis. Less severe side-effects occur in less than 10% of the treated patients and include flu-like symptoms, fatigue, thinning of hair, myalgia, bone marrow suppression requiring dose reduction,

- neuropsychiatric effects, such as depression and autoimmune disease (thyroid). All patients must be carefully monitored by the prescribing doctor for side-effects by using appropriate
- biochemical, haematological and Immunological tests. Appropriate medical records should be maintained.

Newer Search of Antiviral Agents Against Hepatitis B and Hepatitis C

One of these searches for the last two decades has been in the development of a promising antiviral agent against hepatitis B and hepatitis C and other viral infections of liver from the plant, *Phyllanthus amarus*.

Phyllanthus niruri Linn. as it has been indexed in majority of published ethano botanical reviews, until recently, belongs to the family Euphorbiaceae. *Phyllanthus* is one of the largest genera of the family Euphorbiaceae containing about 700 species. It has been shown that about 24 species of *Phyllanthus* are active against clinical Hepatitis (Jaundice) as indicated in Table 2, out of which 8 have been used in India.

Table 2

List of *Phyllanthus* species used in clinical jaundice

(Unander et al., J Ethnopharmacol 1991;34:97-133)

1. *Phyllanthus niruri*
2. *P. amarus*
3. *P. fraternus*
4. *P. mimicus*
5. *P. debilis*
6. *P. urinaria*
7. *P. caroliniensis*
8. *P. abnormis*
9. *P. airy-shavii*
10. *P. tenellus*
11. *P. gastroerni*

12. *P. gunni*
13. *P. similis*
14. *P. thymodes*
15. *P. hirtellus*
16. *P. stipulatus*
17. *P. niruroides*
18. *P. rheedi*
19. *P. acutifolius*
20. *P. hutchinosolianus*
21. *P. cantoniensis*
22. *P. virgatus*
23. *P. corcovadensis*
24. *P. palanensis*

TAXONOMY OF PHYLLANTHUS AMARUS:

This plant has recently been delineated as a mixture of three distinct species namely *Phyllanthus amarus*, *Phyllanthus fraternus* and *Phyllanthus debilis*. It was later identified that the circumtropical weed *P. amarus* is the predominant species in South India, particularly in Tamilnadu. *P. amarus* are erect annual herbs, 10-60 cm tall ; main stem simple or branched, terrete smooth or scabridulous in younger parts. Cataphylls, stipules 1.5-1.9 mm long, deltoid acuminate blade 1-1.5 mm long, subulate acuminate. Deciduous branchlets 1.5-14 cms long, subserate, smooth or a few lower nodes sometimes scabridulous with 13-30 distichous leaves. Leaves 3-11 x 1.5 x 6 mm elliptic oblong obovate, oblong, or even obovate, obtuse, or minutely apiculate at apex, obtuse or slightly inequilateral at base, petioles 0.3-0.5 mm long , stipules 0.8-1.1 mm long triangular acuminate. Flowers in axillary, unisexual and bisexual cymes on deciduous branches. Proximal 2-3 axils with unisexual cymes, each consisting of 1 male and 1 female or 2(-3) males and female or 1 male and 2 female flower or combination thereof ; male flowers pedicels at anthesis ca 1 mm long. Calyx lobe 5, subequal each ca 0.7 x 0.3 mm elliptic or oblong elliptic and abruptly acute at apex hyaline with unbranched mid ribs. Disc segments 5, roundish stamens 3 (rarely 2): filaments connate into a column 0.2-0.3 mm high anthers sessile at top dehiscing longitudinally. Female flowers; pedicels 0.8-1 mm long, obtusely 4 gonous, dilated above, ca 1.5 mm in fruits, calyx 5 lobes, subequal. Ovate-oblong, acute at apex, midsepaline band green. Disc flat, deeply 5 lobed. Lobes sometimes toothed at

apex. Styles 3, free, more or less spreading, shallowly bifid at apex ; arms divergent (Mitra & Jain, Bull Bot Surv Ind 1985;27:167-176).

HISTORICAL USE OF P. NIRURI IN JAUNDICE

Even though clinical uses of *P. niruri* and other species viz. *P. amarus* cited for over a century in the Ayurvedha and Siddha literatures, scientific evaluatory studies have been attempted only during the last 50 years for its efficacy in the treatment of jaundice/viral hepatitis. A logical approach towards identification of the active principles of *P. amarus* is to fractionate the plant extracts and identify biologically active compounds and to chemically characterise them.

STUDIES ON P.NIRURI/P. AMARUS AGAINST HBV:

The first ever designed invitro antiviral study on *Phyllanthus niruri* against any hepatitis virus with HBV as model was reported by Thyagarajan in 1979 from Madras (Thyagarajan, Ph.D. Thesis, University of Madras 1979), India. Subsequently, Thyagarajan et al (1982) have shown the whole plant extract of *P. niruri* through several solvents brought about binding of Hepatitis B surface antigen (HBsAg) (Thyagarajan et al., Ind J Med Res 1982; 76(Suppl.): 124-130. This plant from Tamilnadu, India was later identified taxonomically by Unander as *P. amarus*. Venkateswaran et al (1987) (Proc Natl Acad Sci USA 1989;14:195-201) and Blumberg et al (1989) (Cancer detection and prevention 1987;84:274-278). from United States using the *P. amarus* plants provided by Thyagarajan have shown that the plants collected from Madras, India whose aqueous extracts bound the surface antigen of HBV invitro, have inhibited the viral DNA polymerase (DNAP) of HBV and Woodchuck hepatitis virus (WHV) invitro. When administered intraperitoneally to WHV infected woodchucks, acutely infected animals lost the viral surface antigen ; the surface antigen titre dropped in some chronically infected animals ; the liver cancer rate in treated chronically infected animals was lower than the untreated controls.

Based on these findings they had secured an Australian patent numbered AV-A-56530/86 for a composition of matter useful in the treatment of Hepatitis B virus infection consisting essentially of the methanol extractable components of *P. niruri* L.

Yanagi et al., (Meeting on Hepatitis viruses, Sept 25-28, 1989, Cold Spring Haror Laboratory, NY, 1989,77) from Japan have reported that aqueous extracts of high dilutions of *P. amarus* collected from South India inhibited HBV DNAP, DNAP I, T4-DNAP, the Klenow fragment and reverse transcriptase of avian myeloblastosis virus. Shead et al (1990) (1990 International

symposium on viral Hepatitis and liver diseases, April 4-8, 1990, Houston, TX, USA; A602) from Australia have shown the aqueous extracts to inhibit the endogenous DNAP of DHBV at high dilutions. Niu et al (1990) from Australia in collaboration with Thyagarajan from India using *P. amarus* collected from Madras, Tamilnadu, on treatment of 4-5 week old ducks congenitally infected with Duck hepatitis B virus (DHBV) with suitable controls after a period of 10 weeks treatment showed transient reduction of viral DNA in serum but no effect on the level of virus DNA or surface antigen in the liver (J Med Virol 1990;32:212-218).

Jayaram et al (1996) (Ind J Pathol Microbiol 1996;39(3):211-215) reported invitro inhibition of HBsAg secretion by PLC/PRF/5 (Alexander) cellline for 48 hrs when the cellline was treated with 1 mg/ml concentration of *P. amarus* as a single dose. Lee et al (1996) (European J Clin Invest 1996; 26:1069-76) from USA in collaboration with Thyagarajan have shown that *P. amarus* down-regulates Hepatitis B virus m RNA transcription and replication using transgenic mice and transgenic celllines. The continuation of this collaboration by Ott et al (1997) has shown the cellular and molecular mechanism of HBV suppression by *P. amarus* to be by interrupting interactions between HBV enhancer I and cellular transcription factors (European J Clin Invest 1997; 27:908-915).

The biosafety studies on *P. amarus* dates back to 1971 when Mokkhasmit et al from Thailand using *P. niruri* have reported it to be non toxic to mice at 10 gms/kg body weight (Bull of Dept of Medical Science NAPRALERT Chicago, IL, 1971;12:36-65). Rao (1985) from Andhra Pradesh, India reported 20% aqueous extract of *P. niruri* leaves to be effective to be as an oral pretreatment of 0.2 ml/100 mg body weight against CCl₄ induced hepatotoxicity in rats (Probe 1985;115-119). Syamasundar et al (1985) from Uttar Pradesh, India showed the hexane extracted compounds Phyllanthin and Hypo phyllanthin reduced CCl₄ or galatosamine induced cytotoxicity to cultured rat hepatocytes (J Ethnopharmacol 1985;14:41-44). Jayaram et al (1987) from Madras, India using the aqueous extract of dried whole plant showed no chronic toxicity in mice at 0.2 mg daily dose per animal for 90 days as revealed by physiological, biochemical and histopathological parameters. There was also no cytotoxic or cytotoxic changes when tested in a tissue culture model using vero cell line (Biomedicine

1987;7:9-16). Venkateswaran et al (1987) from USA demonstrated its *in vivo* safety using woodchucks as animal models, while Niu et al (1990) from Australia have shown *P. amarus* to be non toxic in Pekin ducks chronically infected with duck Hepatitis B virus. Jayaram and Thyagarajan (1994) studying the effect of *P. amarus* on β -galactosamine induced hepatotoxicity on isolated rat hepatocytes have shown that a) *P. amarus* by itself did not bring about any hepatotoxicity on rat hepatocytes. b) At 1 mg/ml concentration the aqueous extract were shown to protect isolated rat hepatocytes significantly from β -galactosamine induced hepatotoxicity thus proving the anti hepatotoxic potentials of *P. amarus* (Ind J Med Microbiol 1994;12(4):247-250).

In all the traditional medicine systems, there has been several formulatory medicines for the treatment of jaundice in general without taking into consideration their viral etiology. Even though *P. niruri* was one of the constituents of such medicines, these were always been multitherbal preparations containing anywhere upto 12 medicinal herbs and most of the treatment evaluations were based on clinical improvement only. On the other hand, there is no documented trial report on their use in chronic liver disease patients.

It was in this context, Thyagarajan and his collaborators, after proving the *in vitro* and *in vivo* efficacy and safety of *P. amarus*, conducted 2 open clinical trials in acute viral hepatitis cases and seven clinical trials (2 of them being double blind trials and the others Phase I/II open trials) in chronic carriers of Hepatitis B virus (HBV). Jayanthi et al (1988) (J Gastroenterol and Hepatol 1988; 3:533-534) in a control clinical trial in acute viral hepatitis (AVH) using *P. niruri* on one arm, and other herbal medicines in other groups have shown a significantly greater decrease in transaminases after two weeks treatment with *P. niruri* in both HBsAg positive and negative groups. In a virologically characterised AVH clinical trial, Geetha et al (1992) (J Gen Medicine 1992;4(2):53-58) have shown that a) *P. amarus* treatment has brought about significantly faster biochemical normalcy in both hepatitis A and B ; b) there was a higher rate of HBsAg clearance in *P. amarus* treated AVH-B cases than other treatment modalities and c) there was no observable side effects due to *P. amarus* treatment.

The clinical trials were conducted by Thyagarajan et al between 1988 and 1997. While the first trial 1988 (Lancet 1988; 2:764-766) reported 59% HBsAg clearance in the *P. amarus* treated group, as against 4% in the placebo group, the second open trial (1990) (Lancet 1990;2: 949-950) showed 20% HBsAg clearance and 63.6% loss of infectivity indicated by HBeAg sero-conversion. Parallely, investigators from other countries like Leelarasamee et al

(1990) (Lancet 1990;1:1600-1601) from Thailand, Wang Me Xia et al from China (1991) (Hepatology RLR 1991;21(5):22-24) have reported the non reproducibility of treatment efficacy by the local variety of *P. amarus* grown in their respective countries.

Disclosure of the Invention

CONCEPT & HYPOTHESIS

It was the feeling that the non-reproducibility of treatment efficacy of the extract of the plant *Phyllanthus amarus* was due to the absence in the extract of all the under mentioned antiviral and biological properties which are essentially required for the efficient treatment of Hepatitis B (both acute and chronic) Hepatitis C (both acute and chronic) and other related viral infections of the liver.

- (i) HBsAg binding property facilitating the inactivation of the virus in circulation ultimately leading to viral clearance.
- (ii) HBV-DNA polymerase enzyme inhibiting potential, thus acting as anti-viral preventing the multiplication of HBV.
- (iii) Reverse Transcriptase enzyme inhibition also required for the initiation of HBV replication.
- (iv) Inhibition of HBsAg secretion from HBV transinfected liver cells thus possessing activity against virus infected chronic liver disease conditions.
- (v) Hepatoprotective and antihepatotoxic properties against the liver cell toxicity brought about by all hepatitis viruses (A,B,C,D & E) and other hepatotoxic agents.
- (vi) Immunomodulating property to potentiate the immune system of HBV infected patients towards virus clearance and protective antibody (anti HBs) responses.
- (vii) HCV replication inhibition as shown by converting HCV-RNA positivity to HCV-RNA negativity thus possessing activity against HCV infected chronic liver disease conditions.

In the light of the above mentioned studies coupled with the above mentioned observations, it was necessary to explain the reasons for non-reproducibility of the clinical efficacy of *P. amarus* on one hand and to conduct further clinical trials independently in different places using the *P. amarus* preparation of Thyagarajan. Accordingly clinical trials were conducted on a total of 173 chronic HBV carriers (3 in Chennai (Madras), 1 at Vellore and 1 at Glasgow, UK) subsequent to the earlier two published clinical trials on 98 chronic HBV carriers. The results are presented in Table 3.

Table 3: Summary of seven clinical trials conducted by Thyagarajan and his collaborators on Human HBV carriers using P. amarus grown in Tamilnadu- For convenience it is termed as "University preparation."

Clinical Trial no.	Authors/year	Dosage/ Mgms/tds	Duration	Number treated		HbsAg clearance %		HBeAg sero-conversion %	
				Test	Placebo	Test	Placebo	Test	Placebo
1.	Published Thyagarajan et al (1988)	200	1 m	40	38	59	4	ND	ND
2.	Madras Thyagarajan et al (1990)	250	3 m	20	Nil	20	-	63.6	-
3.	Unpublished Benjamin Samuel et al (1991), Vellore	250	2 m	10	12	20	8.3	37.5	0
4.	Thyagarajan et al (1992), Madras	250	6 m	72	Nil	25	-	54.0	-
5.	Thyagarajan et al (1993), Madras	500	3 m	8	8	25	0	71.4	16.0
6.	Eric Walker et al (1993-95) Glasgow	500	4-6 m	26	Nil	11.6	-	45.4	-
7.	Thyagarajan et al (1996-97), Madras	500	6 m	37	Nil	18.9	-	60.0	-
	Total			213	58	25.6	3.4	55.3	1.7

ND – Not done

In summary, these trials have shown a mean HBsAg clearance rate of 25.6 % and mean HBeAg seroconversion rate of 55.3%. It was finally decided to recommend a schedule of 500 mg dosage of P. amarus preparation in capsules given orally for three times daily for six months.

PHYLLANTHUS AMARUS TREATMENT IN ACUTE AND CHRONIC HEPATITIS C

Available literature in the public domain did not reveal any report on the use of Phyllanthus amarus in the treatment of acute and chronic hepatitis C virus infection which is another major liver pathogen leading to significant morbidity and mortality. Hence the formulation of Phyllanthus amarus envisaged by the present invention was utilised to conduct two clinical trials to treat acute and chronic hepatitis C. Confidential clinical trials have been conducted using the formulation of Phyllanthus amarus in the form of capsules provided by Thyagarajan et al from Chennai. Results are presented in Table 4 and 6.

Since there are no experimental animal/tissue culture models available for testing specific antiviral properties against Hepatitis C virus (HCV), direct studies on human volunteers infected with HCV were conducted. Ethical clearance and informed consents from the study participants were obtained based on the high safety profile of the preparation of P.amarus prepared by the applicants as described above.

Table.4. First set of Case Studies on the efficacy of the preparation of Phyllanthus amarus treatment on chronic hepatitis C infections conducted at Glasgow,UK(Eric Walker et al)

1. Patient having the Date of Birth 07/02/49

Summary : The major finding has been the marked improvement in symptoms when the patient was treated with the preparation of Phyllanthus amarus which symptoms relapsed on 2 occasions when the treatment was withdrawn. The liver enzymes deteriorated when the preparation of Phyllanthus amarus was withdrawn and improved when it was started again. The patient has always been PCR positive. Appearance is of someone who is getting a 'liver protective effect' but without elimination of virus. Source was blood transfusion.

29/01/96- Liver biopsy- some inflammation and hepatic fibrosis consistent with chronic hepatitis C infection. Itch, joint pains, lethargy were the main symptoms . Not keen on interferon- Treatment with the preparation of Phyllanthus amarus was started

23/04/96 – The weight of the patient increased by 2kgs. Markedly improved energy and symptoms. Little change in liver enzymes (AST 42,ALT 50). PCR positive

15/05/96 treatment with the preparation of the composition containing *Phyllanthus amarus* was stopped

17/06/96- Itch, joint pains and lethargy returned (AST 46, ALT 50)

11/07/96 – Treatment with the composition containing *phyllanthus amaraus* was restarted

10/09/96 - Symptoms were much improved (AST 46, ALT 71)

01/07/97 - Still hepatitis C PCR positive(genotype 3). Repeat liver biopsy shows little change (inflammation and fibrosis but no active cirrhotic changes)

05/06/98 – weight of the patient was found to be steady and the patient felt well (AST 42, ALT 59)

08/08/98 – Treatment with the preparation of *Phyllanthus amarus* was stopped

31/12/98 – The patient was found to be depressed and 'run down'(AST 62, ALT 75)

21/01//99 – Treatment with the preparation of *Phyllanthus* restarted following which a marked improvement in the patient 's feeling of well being was observed .

29/12/99 – The patient felt well (AST 50, ALT 69), remained PCR positive

2. Patient having a Date of Birth 20/06/54

Source of HCV infection was intravenous drug use

This patient was one of the first to clear HBsAg and 'e' antigen after treatment with the preparation of *Phyllanthus tamarus* back in 1990 – he remains negative (1999)

He was not tested for hepatitis C until 1999 (April) when he was found to have antibody but was PCR positive. He remained well.

A second set of studies have been carried out by the inventor. Results confirming the efficacy of the new formulation for the treatment of acute and chronic Hepatitis B & C and other related infections of liver are summarised in Table - 6.

THE INVENTION:

We observed that if all the above said six essential properties are made available in a single formulation, the resulting formulation will have uniform and stable antiviral and biological potentials which will be beneficial and optimal for the treatment of acute and chronic hepatitis B & C and other viral diseases of the liver. Based on the above findings, the R & D work for the development of a new formulation of *P. amarus* extract was initiated to find out which formulation will be active significantly against both acute Hepatitis B and against chronic HBV carriers. Accordingly, R & D was directed towards developing such a formulation from the plant *Phyllanthus amarus* containing all the above said essential characteristics which have not only efficient clinical and biological efficacy against acute and chronic Hepatitis B; acute and chronic Hepatitis C and other viral infections of the liver but also have uniform and stable antiviral and bio active properties.

Objectives of the invention

Accordingly, the main objective of the present invention, therefore, is to provide a pharmaceutical formulation having uniform and stable antiviral and biological efficacy which is useful for the treatment of acute and chronic Hepatitis B, Hepatitis C, chronic HIV carriers and other related viral infections of the liver from the plant, *Phyllanthus amarus*

Another objective of the present invention is to provide a pharmaceutical formulation useful for the treatment of Hepatitis B, Hepatitis C, chronic HIV carriers and other related viral infections of the liver from the plant *Phyllanthus amarus* which brings about binding of Hepatitis B surface antigen (HBsAg) of the Hepatitis B virus, thus facilitating the inactivation of the virus in circulation ultimately leading to viral clearance.

Yet another objective of the present invention is to provide a pharmaceutical formulation useful for the treatment of Hepatitis B, Hepatitis C, chronic HIV carriers and other related viral infections of the liver from the plant *P. amarus* which inhibit the HBV-DNA polymerase enzyme required for the replication for the virus, thus acting as antiviral preventing the multiplication of the virus itself.

Still another objective of the present invention is to provide a pharmaceutical formulation useful for the treatment of Hepatitis B, Hepatitis C, chronic HIV carriers and other related viral infections of the liver from the plant *P. amarus* which inhibits the Reverse Transcriptase enzyme which is also required for the initiation of HBV replication and is the chief enzyme required for the replication of the AIDS virus, Human Immunodeficiency virus (HIV).

Another objective of the present invention is to provide a process for the preparation of a pharmaceutical formulation useful for the treatment of Hepatitis B, Hepatitis C, chronic HIV carriers and other related viral infections of the liver from the plant *P. amarus* which is hepatoprotective and also possess antihepatotoxic properties against the liver cell toxicity brought about by all Hepatitis viruses (A,B,C,D & E) and other hepatotoxic agents including chemicals and aflatoxins.

Yet another objective of the present invention is to provide a pharmaceutical formulation useful for the treatment of acute and chronic Hepatitis B; acute and chronic Hepatitis C, chronic HIV carriers and other related viral infections of the liver from the plant *P. amarus* which is anti-inflammatory and also possesses the property of normalising the transaminase enzymes level indicating antihepatotoxic and liver cell regenerating potentials of the formulation.

Still another objective of the present invention is to provide a pharmaceutical formulation useful for the treatment of acute and chronic Hepatitis B; acute and chronic Hepatitis C, chronic HIV carriers and other related viral infections of the liver from plant *P. amarus* which inhibited the Hepatitis C virus replication as revealed by conversion from HCV-RNA positivity to HCV-RNA negativity in patients treated by the formulation.

Another objective of the present invention is to provide a pharmaceutical formulation useful for the treatment of acute and chronic Hepatitis B; acute and chronic Hepatitis C, chronic HIV carriers and other related viral infections of the liver from plant *P. amarus* which is immunomodulatory as revealed by increased T-cell proliferation.

Still another objective of the present invention is to provide a pharmaceutical formulation useful for the treatment of acute and chronic Hepatitis B, acute and chronic Hepatitis C and other related viral infections of the liver from the plant *P. amarus* in which the antiviral and biological activities are uniform

Yet another objective of the present invention is to provide a process for the preparation of a pharmaceutical formulation useful for the treatment of acute and chronic Hepatitis B acute and chronic Hepatitis C and other related viral infections of the liver from the plant *P. amarus* with antihepatotoxic, liver cell regenerating and immunomodulating potentials.

The invention is based on our surprising findings that when the parts of the plant *Phyllanthus amarus* are extracted separately with a polar solvent alone, mixture of polar solvent and water and water alone and when the extracts so obtained are mixed together, the resultant formulation is found to have all the under mentioned properties which are required for the efficient treatment of Hepatitis B (both acute and chronic), Hepatitis C (both acute and chronic) and other related viral infections of the liver.

- (i) HBsAg binding property facilitating the inactivation of the virus in circulation ultimately leading to viral clearance.
- (ii) HBV-DNA polymerase enzyme inhibiting potential, thus acting as anti-viral, preventing the multiplication of HBV.
- (iii) Reverse Transcriptase enzyme inhibition also required for the initiation of HBV replication.
- (iv) Inhibition of HBsAg secretion from HBV transinfected liver cells thus possessing activity against virus infected chronic liver disease conditions.
- (v) Hepato-protective, anti-inflammatory, anti-hepatotoxic and liver cell regenerative properties against the liver cell toxicity brought about by all hepatitis viruses (A,B,C,D & E) and other hepatotoxic agents.
- (vi) Immunomodulating property to potentiate the immune system of HBV infected patients towards virus clearance and protective antibody (anti HBs) responses.
- (vii) HCV-replication inhibition as shown by converting HCV-RNA positivity to HCV-RNA negativity thus possessing activity against HCV infected chronic liver disease conditions.

It is observed that the individual extracts of the plant *Phyllanthus amarus* namely the extract obtained by using a polar solvent alone, extract obtained by using a polar solvent and water and the extract obtained by using water alone, themselves, does not possess all the above said essential characteristics. But the combination of the above mentioned extracts imparts to the resulting formulation all the above mentioned essential properties which are required for the efficient treatment of Hepatitis B (both acute and chronic), Hepatitis C (both acute and

chronic) and other related viral infections of the liver. These properties are acquired by the formulation due to the biological synergism of the different components contained in the individual extracts when combined to form the formulation.

The pharmaceutical formulation of the present invention is not, therefore, a mere admixture of the individual components resulting in the aggregation of the properties of the individual components but is a novel pharmaceutical formulation having biological synergism of all the required efficacious antiviral and biological properties of the components employed.

Accordingly, the present invention provides a pharmaceutical formulation useful for the treatment of Hepatitis B (both acute and chronic) Hepatitis C (both acute and chronic) and other related viral infections of the liver which comprises

- (i) an extract of the plant *Phyllanthus amarus* obtained by using a polar solvent alone
- (ii) an extract of the plant *Phyllanthus amarus* obtained by using a mixture of a polar solvent and water and
- (iii) an extract of the plant *Phyllanthus amarus* obtained by using water alone

According to another feature, the present invention provides a pharmaceutical formulation useful for the treatment of Hepatitis B (both acute and chronic), Hepatitis C (both acute and chronic) and other related viral infections of the liver which comprises

- (i) 10 to 40% w/w of an extract of the plant *Phyllanthus amarus* obtained by using a polar solvent alone
- (ii) 10 to 40% w/w of an extract of the plant *Phyllanthus amarus* obtained by using a mixture of a polar solvent and water wherein the ratio of the solvent to water ranges from 20 to 80 and 80 to 20% w/w respectively and
- (iii) 10 to 40% w/w of an extract of the plant *Phyllanthus amarus* obtained by using water alone

In another preferred embodiment of the invention, there is provided a pharmaceutical formulation useful for the treatment of Hepatitis B (both acute and chronic), Hepatitis C (both acute and chronic) and other related viral infections of the liver which comprises

- (i) 20 to 30% w/w of an extract of the plant *Phyllanthus amarus* obtained by using a polar solvent alone
- (ii) 20 to 40% w/w of an extract of the plant *Phyllanthus amarus* obtained by using a mixture of a polar solvent and water wherein the ratio of the solvent to water ranges from 80 to 20% and 20 to 80% w/w respectively
- (iii) 20 to 40-% w/w of an extract of the plant *Phyllanthus amarus* obtained by using a polar solvent and water wherein the solvent to water ratio ranges from 20 to 80 % and 80 to 20 % w/w respectively and
- (iv) 20 to 30% w/w of an extract of the plant *Phyllanthus amarus* obtained by using water alone

In still another preferred embodiment of the present invention there is provided a pharmaceutical formulation useful for the treatment of Hepatitis B (both acute and chronic), Hepatitis C (both acute and chronic) and other related viral infections of the liver which comprises **one part each of** an extract of the plant *Phyllanthus amarus* obtained by using a polar solvent alone , an extract of the plant *Phyllanthus amarus* obtained by using a mixture of a polar solvent and water and an extract of the plant *Phyllanthus amarus* using water alone.

In yet another preferred embodiment of the present invention, there is provided a pharmaceutical formulation useful for the treatment of Hepatitis B (both acute and chronic), Hepatitis C (both acute and chronic) and other related viral infections of the liver which comprises **one part each of**

- (i) 10 to 40% w/w of an extract of the plant *Phyllanthus amarus* obtained by using a polar solvent alone.
- (ii) 10 to 40% w/w of an extract of the plant *Phyllanthus amarus* obtained by using a mixture of a polar solvent and water wherein the ratio of the solvent to water ranges from 20 to 80 and 80 to 20% w/w respectively and
- (iii) 10 to 40% w/w of an extract of the plant *Phyllanthus amarus* obtained by using water alone.

In another preferred embodiment of the present invention, there is provided a pharmaceutical formulation useful for the treatment of Hepatitis B (both acute and chronic), Hepatitis C (both

acute and chronic) and other related viral infections of the liver which comprises **one part each of**

- (i) 20 to 30 %w/w of an extract of the plant *Phyllanthus amarus* obtained by using a polar solvent alone ,
- (ii) 20 to 40 %w/w of an extract of the plant *Phyllanthus amarus* obtained by using a mixture of a polar solvent and water wherein the ratio of the solvent to water ranges from 80 to 20% and 20 to 80 % w/w respectively
- (iii) 20 to 40 % w/w of an extract of the plant *Phyllanthus amarus* using a mixture of a polar solvent and water wherein the ratio of the solvent to water ranges from 20 to 80 % w/w and 80 to 20 % w/w respectively and
- (iv) 20 to 30 % w/w of an extract obtained using water alone

In still another preferred embodiment of the present invention, there is provided a pharmaceutical formulation useful for the treatment of Hepatitis B (both acute and chronic), Hepatitis C (both acute and chronic) and other related viral infections of the liver which comprises **two parts**

- (i) of an extract of the plant *Phyllanthus amarus* obtained by using a polar solvent alone and **one part each of**
- (ii) of an extract of the plant *Phyllanthus amarus* obtained by using a mixture of a polar solvent and water and
- (iii) an extract of the plant *Phyllanthus amarus* using water alone

In yet another preferred embodiment of the present invention, there is provided a pharmaceutical formulation useful for the treatment of Hepatitis B (both acute and chronic), Hepatitis C (both acute and chronic) and other viral infections of the liver which comprises **two parts**

- (i) of 10 to 40 %w/w of an extract of the plant *Phyllanthus amarus* obtained by using a polar solvent **and one part each**
- (ii) of 10 to 40 % w/w of an extract of the plant *Phyllanthus amarus* obtained by using a mixture of a polar solvent and water wherein the ratio of the solvent to water ranges from 20 to 80% and 80 to 20 % w/w respectively
- (iii) of 10 to 40% w/w of an extract of the plant *Phyllanthus amarus* obtained by using water alone

In yet another preferred embodiment of the present invention there is provided a pharmaceutical formulation useful for the treatment of Hepatitis B (both acute and chronic), Hepatitis C (both acute and chronic) and other viral infections of the liver which comprises **two parts**

- (i) of 20 to 30 % W/W of an extract of the plant *Phyllanthus amarus* obtained by using a polar solvent alone **and one part each**
- (ii) of 20 to 40 % w/w of an extract of the plant *Phyllanthus amarus* obtained by using a mixture of a polar solvent and water wherein the ratio of the solvent to water ranges from 80 to 20% and 20 to 80 % w/w respectively
- (iii) of 20 to 40 % w/w of an extract of the plant *Phyllanthus amarus* obtained by using a mixture of polar solvent and water wherein the ratio of the solvent to water ranges from 20 to 80% w/w and 80 to 20% w/w respectively and .
- (iv) of 20 to 30 % w/w of an extract of the plant *Phyllanthus amarus* obtained by using water alone.

In yet another preferred embodiment of the invention, there is provided a process for the preparation of pharmaceutical formulation useful for the treatment of Hepatitis B (acute and chronic), Hepatitis C (both acute and chronic) and other related viral infections of the liver which comprises mixing

- (i) an extract of the plant *Phyllanthus amarus* obtained by using a polar solvent alone
- (ii) an extract of the plant *Phyllanthus amarus* obtained by using a mixture of a polar solvent and water and
- (iii) an extract of the plant *Phyllanthus amarus* obtained by using water alone

According to another feature the present invention, provides a process for preparing a pharmaceutical formulation useful for the treatment of Hepatitis B (both acute and chronic), Hepatitis C (both acute and chronic) and other related viral infections of the liver which comprises mixing

- (i) 10 to 40% w/w of an extract of the plant *Phyllanthus amarus* obtained by using a polar solvent alone
- (ii) 10 to 40% w/w of an extract of the plant *Phyllanthus amarus* obtained by using a mixture of a polar solvent and water wherein the ratio of the solvent to water ranges from 20 to 80 and 80 to 20% w/w respectively and
- (iii) 10 to 40% w/w of an extract of the plant *Phyllanthus amarus* obtained by using water alone

In another preferred embodiment of the invention, there is provided a process for the preparation of a pharmaceutical formulation useful for the treatment of Hepatitis B (both acute and chronic), Hepatitis C (both acute and chronic) and other related viral infections of the liver which comprises mixing

- (i) 20 to 30% w/w of an extract of the plant *Phyllanthus amarus* obtained by using a polar solvent alone
- (ii) 20 to 40% w/w of an extract of the plant *Phyllanthus amarus* obtained by using a mixture of a polar solvent and water wherein the ratio of the solvent to water ranges from 80 to 20% and 20 to 80% w/w respectively
- (iii) 20 to 40% w/w of an extract of the plant *Phyllanthus amarus* obtained by using a polar solvent and water wherein the solvent to water ratio ranges from 20 to 80 % and 80 to 20 % w/w respectively and
- (iv) 20 to 30% w/w of an extract of the plant *Phyllanthus amarus* obtained by using water alone

In still another preferred embodiment of the present invention, there is provided a process for the preparation of pharmaceutical formulation useful for the treatment of Hepatitis B (both acute and chronic), Hepatitis C (both acute and chronic) and other related viral infections of the liver comprises mixing **one part each of**

- (i) an extract of the plant *Phyllanthus amarus* obtained by using a polar solvent alone ,
- (ii) an extract of the plant *Phyllanthus amarus* obtained by using a mixture of a polar solvent and water and
- (iii) an extract of the plant *Phyllanthus amarus* using water alone

In yet another preferred embodiment of the present invention, there is provided a process for the preparation of pharmaceutical formulation useful for the treatment of Hepatitis B (both acute and chronic), Hepatitis C (both acute and chronic) and other related viral infections of the liver which comprises mixing **one part each of**

- (i) 10 to 40% w/w of an extract of the plant *Phyllanthus amarus* obtained by using a polar solvent alone.
- (ii) 10 to 40% w/w of an extract of the plant *Phyllanthus amarus* obtained by using a mixture of a polar solvent and water wherein the ratio of the solvent to water ranges from 20 to 80 and 80 to 20% w/w respectively and
- (iii) 10 to 40% w/w of an extract of the plant *Phyllanthus amarus* obtained by using water alone

In another preferred embodiment of the present invention there is provided a process for the preparation of a pharmaceutical formulation useful for the treatment of Hepatitis B (both acute and chronic), Hepatitis C (both acute and chronic) and other related viral infections of the liver which comprises mixing **one part each of**

- (i) 20 to 30 %w/w of an extract of the plant *Phyllanthus amarus* obtained by using a polar solvent alone,
- (ii) 20 to 40 %w/w of an extract of the plant *Phyllanthus amarus* obtained by using a mixture of a polar solvent and water wherein the ratio of the solvent to water ranges from 80 to 20% and 20 to 80 % w/w respectively
- (iii) 20 to 40 % w/w of an extract of the plant *Phyllanthus amarus* using a mixture of a polar solvent and water wherein the ratio of the solvent to water ranges from 20 to 80 % w/w and 80 to 20 % w/w respectively and
- (iv) 20 to 30 % w/w of an extract obtained using water alone

In still another preferred embodiment of the present invention, there is provided a process for the preparation of a pharmaceutical formulation useful for the treatment of Hepatitis B (both acute and chronic), Hepatitis C (both acute and chronic) and other related viral infections of the liver which comprises mixing **two parts**

- (i) of an extract of the plant *Phyllanthus amarus* obtained by using a polar solvent alone and **one part each of**
- (ii) of an extract of the plant *Phyllanthus amarus* obtained by using a mixture of a polar solvent and water and
- (iii) an extract of the plant *Phyllanthus amarus* using water alone

In yet another preferred embodiment of the present invention, there is provided a process for the preparation of a pharmaceutical formulation useful for the treatment of Hepatitis B (both acute and chronic), Hepatitis C (both acute and chronic) and other viral infections of the liver which comprises **mixing two parts**

- (i) of 10 to 40 %w/w of an extract of the plant *Phyllanthus amarus* obtained by using a polar solvent and **one part each**
- (ii) of 10 to 40 % w/w of an extract of the plant *Phyllanthus amarus* obtained by using a mixture of a polar solvent and water wherein the ratio of the solvent to water ranges from 20 to 80% and 80 to 20 % w/w respectively
- (iii) of 10 to 40% w/w of an extract of the plant *Phyllanthus amarus* obtained by using water alone

In yet another preferred embodiment of the present invention, there is provided a process for the preparation pharmaceutical formulation useful for the treatment of Hepatitis B (both acute and chronic), Hepatitis C (both acute and chronic) and other viral infections of the liver which comprises mixing **two parts**

- (i) of 20 to 30 % W/W of an extract of the plant *Phyllanthus amarus* obtained by using a polar solvent alone and **one part each**
- (ii) of 20 to 40 % w/w of an extract of the plant *Phyllanthus amarus* obtained by using a mixture of a polar solvent and water wherein the ratio of the solvent to water ranges from 80 to 20% and 20 to 80 % w/w respectively
- (iii) of 20 to 40 % w/w of an extract of the plant *Phyllanthus amarus* obtained by using a mixture of polar solvent and water wherein the ratio of the solvent to water ranges from 20 to 80% w/w and 80 to 20% w/w respectively and .
- (iv) of 20 to 30 % w/w of an extract of the plant *Phyllanthus amarus* obtained by using water alone.

The present invention also envisages within its scope the use of the pharmaceutical composition of the present invention described above for the preparation of a medicament useful for treating Hepatitis B (both acute and chronic), Hepatitis C (both acute and chronic) and other related viral infections of the liver.

The invention further envisages the use of the pharmaceutical composition of the present invention in the treatment of Hepatitis B (both acute and chronic), Hepatitis C (both acute and chronic) and other related viral infections of the liver.

Furthermore the present invention also, within its scope includes, the use of the pharmaceutical composition described above for achieving antihepatotoxicity, liver cell regeneration and immunomodulation.

In another embodiment of the present invention there is provided a method of treating Hepatitis B (both acute and chronic), Hepatitis C (both acute and chronic) and other related viral infections of the liver which comprises administering a therapeutically effective dose of the pharmaceutical composition described above.

Extraction of the components of the formulation:

Parts such as leaves, stems, seeds and roots of the taxonomically identified collections of the plant *Phyllanthus amarus* were dried and kept in an oven heated to a temperature in the range of 50 to 80 degree C for a period in the range of 3 to 5 hrs a day. for a period of 3 to 6 successive days. The dried parts of the plants are then powdered. The powder thus obtained is used for the extraction of different components of the preparation of the formulation of the present invention.

Before subjecting the powder for extraction procedures, each batch of powder is subjected for sterility testing to rule out any bacterial or fungal contamination as per standard methods.

One portion of the powder is extracted with polar solvent. The polar solvent employed may be methanol, ethanol, hexane, butanol and the like. The extraction may be carried out at a temperature in the range of 37 to 60 degree C for a period ranging from 2 hours to 18 hours preferably at a temperature in the range of 37 to 60 degree C. This extract may be used as the component (i) of the pharmaceutical formulation of the present invention.

Another portion of the powder is extracted with a mixture of polar solvent and water. The polar solvent employed may be methanol, ethanol, Hexane and the like. The ratio of the solvent and water used for extraction may range from 20 to 80 and 80 to 20% w/w respectively, The ratio may preferably range from 30 to 50% and 50 to 30% w/w respectively. The extraction may be carried out at a temperature in the range of 4 to 40 degree C for 2 to 18 hours more preferably at a temperature in the range of 37 to 60 degree C for 2 to 4 hours. This extract may be used as the component (ii) of the pharmaceutical formulation of the present invention.

Yet another portion of the powder is extracted with water alone. The extraction may be carried out at a temperature in the range of 37 to 60 degree C for 2 to 18 hours. This extract may be used as the component (iii) of the pharmaceutical formulation of the invention

The extracts so obtained may now be mixed together to obtain the pharmaceutical formulation of the present invention. The mixing may be effected in a vortex mixer or heating mantle and stirring it thoroughly till a homogenous formulation is obtained. The mixing may be effected at a temperature in the range of 37 to 60 degree C for 15 to 30 minutes.

The details of the invention are given in the Examples provided below which are given to illustrate the invention only and therefore should not be construed to limit the scope of the present invention.

Example 1

40 gms of the powder obtained as explained above is extracted with 200 ml of ethanol at a temperature of 35 to 37 degree C for 2 hrs and 56 degree C for another 2 hrs under shaking. Yield of the extract is 90ml (EXTRACT-I)

40 gms of the powder obtained as explained above is extracted with a mixture of 160 ml of ethanol and 40 ml water total volume being 200 ml. The extraction was effected at a temperature of 35 to 37 degree C for 2 hrs and 56-60 degree C for another 2 hrs under shaking. Yield of the extract 65 ml (EXTRACT-II)

40 gms of the powder prepared as explained above is extracted with 200 ml water at temperature of 35 to 37 degree C for 2 hrs and 56-60 degree C for another 2 hrs under shaking. Yield of the extract 50 ml (EXTRACT-III)

Extracts I, II, and III were mixed in 1:1:1 ratio (i.e., 50 ml each) in a conical flask in an environmental shaker kept at 37°C for 15-30 min. The resultant formulation was dried in a dessicator/"Vertis" vaccum drier until the solvents get fully evaporated. The yield of the powdery formulation was 28 gms (20%).

Example 2

40 gms of the powder obtained as explained above is extracted with 200 ml of ethanol at a temperature of 35 to 37 degree C for 2 hrs and 56-60 degree C for another 2 hrs under shaking. Yield of the extract 90 ml (EXTRACT-I)

40 gms of the powder obtained as explained above is extracted with a mixture of 160 ml of ethanol and 40 ml water total volume being 200ml. The extraction was effected at a temperature of 35 to 37 degree C for 2 hrs and 56-60 degree C for another 2 hrs under shaking. Yield of the extract 65 ml (EXTRACT-II)

40 gms of the powder prepared as explained above is extracted with 200ml water at temperature of 35 to 37 degree C for 2 hrs and 56-60 degree C for another 2 hrs under shaking. Yield of the extract 50 ml (EXTRACT-III)

The extracts I, II & III in 2:1:1 ratio (i.e., 80 ml of extract I: 40ml of extract II and 40ml of extract III) were mixed in a conical flask in an environmental shaker kept at 37°C for 15-30 min. The resultant formulation was dried in a dessicator/"Vertis" vaccum drier until the solvents get fully evaporated. The final yield when dried was 36 gms (25%).

Example 3

30 gms of the powder obtained as explained above is extracted with 200 ml of methanol at a temperature of 35 to 37 degree C for 2 hrs and 56-60 degree C for another 2 hrs under shaking. Yield of the extract 90 ml (EXTRACT-I)

30 gms of the powder obtained as explained above are extracted with a mixture of 60 ml of methanol and 140 ml water. The extraction was effected at a temperature of 35 to 37 degree C for 2 hrs and 56-60 degree C for another 2 hrs under shaking. Yield of the extract 70 ml (EXTRACT-II)

30 gms of the powder prepared as explained above is extracted with 200 ml water at temperature of 35 to 37 degree C for 2 hrs and 56-60 degree C for another 2 hrs under shaking. Yield of the extract 50 ml (EXTRACT-III)

The Extracts I, II and III were mixed in 1:1:1 ratio (i.e., 50 ml each) in a conical flask in an environmental shaker kept at 37°C for 15-30 min. The resultant formulation was dried in a dessicator/"Vertis" vaccum drier until the solvents get fully evaporated. The yield of the powdery formulation was 25 gms (15%).

Example 4

30 gms of the powder obtained as explained above is extracted with 200 ml of methanol at a temperature of 35 to 37 degree C for 2 hrs and 56-60 degree C for another 2 hrs under shaking. Yield of the extract 90 ml (EXTRACT-I)

30 gms of the powder obtained as explained above are extracted with a mixture of 60 ml of methanol and 140 ml water. The extraction was effected at a temperature of 35 to 37 degree C for 2 hrs and 56-60 degree C for another 2 hrs under shaking. Yield of the extract 70 ml (EXTRACT-II)

30 gms of the powder prepared as explained above is extracted with 200 ml water at temperature of 35 to 37 degree C for 2 hrs and 56-60 degree C for another 2 hrs under shaking. Yield of the extract 50 ml (EXTRACT-III)

The extracts I, II and III in 2:1:1 ratio (i.e., 80 ml of extract I: 40 ml of II and 40 ml of III) were mixed in a conical flask in an environmental shaker kept at 37°C for 15-30 min. The resultant formulation was dried in a dessicator/"Vertis" vaccum drier until the solvents get fully evaporated. The final yield was 32 gms (22%).

Example 5

40 gms of the powder obtained as explained above is extracted with 200 ml of methanol at a temperature of 56 to 60 degree C for 4 hrs under shaking. Yield of the extract 90 ml (EXTRACT-I)

35 gms of the powder obtained as explained above is extracted with a mixture of 100 ml of methanol and 100 ml water, total volume being 200 ml. The extraction was effected at a temperature of 35 to 37 degree C for 2 hrs and 56-60 degree C for another 2 hrs under shaking. Yield of the extract 70 ml (EXTRACT-II)

30 gms of the powder prepared as explained above is extracted with 200 ml water at temperature of 56 to 60 degree C for 4 hrs under shaking. Yield of the extract 60 ml (EXTRACT-III)

Extracts I, II and III were mixed in 1:1:1 ratio (i.e., 50 ml each) in a conical flask in an environmental shaker kept at 37°C for 15-30 min. This formulation was dried in a dessicator/"Vertis" vacuum drier until the solvents get fully evaporated. The yield of the powdery formulation so obtained was 25 gms (15%).

Example 6

40 gms of the powder obtained as explained above is extracted with 200 ml of methanol at a temperature of 56 to 60 degree C for 4 hrs under shaking. Yield of the extract 90 ml (EXTRACT-I).

35 gms of the powder obtained as explained above is extracted with a mixture of 100 ml of methanol and 100 ml water, total volume being 200 ml. The extraction was effected at a temperature of 35 to 37 degree C for 2 hrs and 56-60 degree C for another 2 hrs under shaking. Yield of the extract 70 ml (EXTRACT-II)

30 gms of the powder prepared as explained above is extracted with 200 ml water at temperature of 56 to 60 degree C for 4 hrs under shaking. Yield of the extract 60 ml (EXTRACT-III)

The Extracts I, II, III were mixed in 2:1:1 ratio (i.e., 80 ml of extract I: 40 ml of extract II and 40 ml of extract III) were mixed in a conical flask in an environmental shaker kept at 37°C for 15-30 min. This formulation was dried in a dessicator/"Vertis" vaccum drier until the solvents get fully evaporated. The final yield when dried was 40 gms (25%).

Example 7

40 gms of the powder obtained as explained above is extracted with 200 ml of methanol at a temperature of 35 to 37 degree C for 2 hrs and 56-60 degree C for another 2 hrs under shaking. Yield of the extract 90 ml (EXTRACT-I)

40 gms of the powder prepared as explained above is extracted with 200 ml water at a temperature of 35 to 37 degree C for 2 hrs and 56-60 degree C for another 2 hrs under shaking. Yield of the extract 60 ml (EXTRACT-II)

35 gms of the powder obtained as explained above is extracted with a mixture of 100 ml of methanol and 100 ml water, total volume being 200 ml. The extraction was effected at a temperature of 56 to 60 degree C for 4 hrs under shaking. Yield of the extract 70 ml (EXTRACT-III)

30 gms of the powder obtained as explained above is extracted with a mixture of 60 ml of methanol and 140 ml water total volume being 200 ml. The extraction was effected at a temperature of 56 to 60 degree C for 4 hrs shaking. Yield of the extract 65 ml (EXTRACT-IV)

The Extracts I, II, III and IV were mixed in 1:1:1:1 ratio (i.e., 50 ml each) in a conical flask in an environmental shaker kept at 37°C for 15-30 min. The resultant formulation was dried in a dessicator/"Vertis" vaccum drier until the solvents get fully evaporated. The yield of the powdery formulations so obtained was 40 gms (25%).

Example 8

40 gms of the powder obtained as explained above is extracted with 200 ml of methanol at a temperature of 35 to 37 degree C for 2 hrs and 56-60 degree C for another 2 hrs under shaking. Yield of the extract 90 ml (EXTRACT-I)

40 gms of the powder prepared as explained above is extracted with 200 ml water at a temperature of 35 to 37 degree C for 2 hrs and 56-60 degree C for another 2 hrs under shaking. Yield of the extract 60 ml (EXTRACT-II)

35 gms of the powder obtained as explained above is extracted with a mixture of 100 ml of methanol and 100 ml water, total volume being 200 ml. The extraction was effected at a temperature of 56 to 60 degree C for 4 hrs under shaking. Yield of the extract 70 ml (EXTRACT-III)

30 gms of the powder obtained as explained above is extracted with a mixture of 60 ml of methanol and 140 ml water total volume being 200 ml. The extraction was effected at a temperature of 56 to 60 degree C for 4 hrs shaking. Yield of the extract 65 ml (EXTRACT-IV)

The extracts I, II, III & IV were mixed in 2:1:1:1 ratio (i.e., 80 ml of extract I: 40 ml of extract II, 40 ml of extract III and 40 ml of extract IV) were mixed in a conical flask in an environmental shaker kept at 37°C for 15-30 min. The resultant formulation was dried in a dessicator/"Vertis" vaccum drier until the solvents get fully evaporated. The final yield when dried was 48 gms (30%).

The examples given below are provided to compare the properties of the extract of the plant *Phyllanthus amarus* obtained using polar solvent alone and water alone with the properties of the pharmaceutical formulation of the present invention.

Example 9

40 gms of the powder obtained as explained above is extracted with 200 ml of methanol at a temperature of 35 to 37 degree C for 2 hrs and 56-60 degree C for another 2 hrs under shaking. Yield of the extract 90 ml.

This extract as a formulation was dried in a dessicator/"Vertis" vacuum drier until the solvents get fully evaporated. The yield of the powdery formulations so obtained was 15 gms (12.5%).

Example 10

40 gms of the powder prepared as explained above is extracted with 200 ml water at a temperature of 35 to 37 degree C for 2 hrs and 56-60 degree C for another 2 hrs under shaking. Yield of the extract 50 ml This extract was dried in a dessicator/"Vertis" vacuum drier until the water get fully evaporated. The yield of the powder so obtained was 8 gms (10%).

BIOLOGICAL EFFICACY OF THE PHARMACEUTICAL FORMULATION WITH PARTICULAR REFERENCE TO THE FORMULATION OBTAINED BY THE PROCESS DESCRIBED IN EXAMPLES 1 to 10

The powdery formulations obtained in the Examples 1 to 10 were used for the biological studies to confirm the presence of the above mentioned essential properties.

For these studies, Phosphate buffered saline (PBS) as vehicle in w/v concentration was employed.

The solutions of the formulations prepared as mentioned above were used for the following assay methods for confirming the presence of the essential properties mentioned above

A. HBSAG BINDING PROPERTY OF THE PHARMACEUTICAL FORMULATION OF THE INVENTION

Principle: The test is an enzyme immunoassay based on the sandwich principle. HBsAg in the plasma is neutralised or bound by pre-incubation with anti-HBs like substance, and hence no longer reacts with the antibody coated in the wells. The presence of HBsAg binding activity in the formulation of the present invention is demonstrated by reduction of colour or a negative ELISA result. The presence of unbound HBsAg in a test sample is demonstrated by an increase in the colour or a positive ELISA result.

Procedure: Pre-incubation with HBsAg plasma: Equal volume of pre-titrated HBsAg positive plasma and 5 mg/ml concentration of the formulation of the present invention was mixed and incubated at 37°C for 5 days. The mixture was assayed daily for the presence of bound/unbound HBsAg using Hepanostika (Organon) or any other commercial ELISA kit. Control tubes containing solvent (PBS) and plasma (solvent control) and P. amarus and plasma

(positive control) were set up in each batch. ELISA was performed as per the instructions of the manufacturer given in the kit.

Calculation:

1. Cut-off value: the cut-off value was calculated as mean negative control+ 0.025
2. Values equal to or greater than the cut-off should be taken as positive

The results of the assays of the pharmaceutical formulation of the present invention prepared by the process described in the Examples 1 to 8 are given in Table 5.

B. HBV – DNA polymerase inhibiting properties of the pharmaceutical formulation of the invention

Principle: Replication of Hepadna viruses involves a viral DNA polymerase which is a potential target for chemotherapy against HBV. In the presence of HBV-DNA polymerase, complementary bases are added to the template (HBV-DNA), the addition of which are quantitated with the help of tritiated thymidine triphosphate. Reduction in the count of 50% or more in the test is noted as inhibitory activity.

Virus preparation: Pre-titrated HBsAg and HBeAg positive serum was centrifuged at 35,000 rpm for 3 hrs using SW 41 rotor. The pellet was washed in PBS and again centrifuged at 35,000 rpm. The pellet got in this was dissolved in PBS and stored at -20°C.

Procedure: The procedure followed was as described by Lofgren et al (1989). Prior to the assay, the virus preparation was pre-treated with 1/8 volume of 2% mercapto ethanol and 10% NP-40 for 15-30 min at room temperature. Aliquotes of 25 µl were then incubated at 37°C for 3 hrs together with 25 µl reaction mixture containing Tris-HCl (pH 8.0) 100mM, MgCl₂ 20mM, KCl 200mM, dNTPs 10mM each and ³H dTTP 10mM and 25µl of DNase and RNase free water and the formulation of the present invention which is to be studied. After incubation, to 50µl of reaction mixture 10µl of 0.2M EDTA was added and spotted onto a Whatman DE81 filter paper discs and processed for a radioactivity measurement.

The results of the assays of the pharmaceutical formulation of the present invention prepared by the process described in the Examples 1 to 8 are given in Table 5.

C. REVERSE TRANSCRIPTASE INHIBITING PROPERTY OF THE PHARMACEUTICAL FORMULATION OF THE INVENTION

This property was evaluated by Electrophoretic and Isotopic RT-Inhibition assays.

ELECTROPHORETIC RT-INHIBITION ASSAY

The electrophoretic RT-inhibition was conducted to screen and identify the anti-retroviral potentials of formulations of the present invention prepared by the process described in Examples 1 to 8. Moloney murine leukaemia virus reverse transcriptase (MMLV RT) was used for cDNA synthesis.

Principle: Reverse transcriptase (RNA-dependent DNA polymerase) is an enzyme in retroviruses and plays an important role in their multiplication by transcribing the viral RNA into cDNA which is required for the proviral synthesis. This test is performed to find the ability of an extract to inhibit RT. The presence of this property is determined by the formation or non-formation of cDNA.

Procedure: The electrophoretic RT –inhibition assay was performed in a reaction mixture that contained the following in a final volume of 25 µl. Tris HCl (pH 8.3) 50 mM, MgCl₂ 6mM, KCl 40mM, P(rA)(dT)₁₂₋₁₈ 0.5 µg, MMLV RT 5 Units. To this 2 µl of the formulation of the present invention prepared by the process described in the Examples 1 to 10 was added immediately before incubation. A positive control and solvent control were set up using Azidothymidine (1 µg/ml) and the solvent used for reconstitution of extract, respectively. All the tubes were incubated at 37°C for one hour, and the reaction was stopped using 0.1 M EDTA. 10 µl of each assay mix was loaded onto a 1% gel and run for 30 minutes at 60 V. The gel was stained in Ethidium bromide (0.5 µg/ml) and viewed in UV transilluminator. The presence of absence of the cDNA band indicates the non-inhibition of inhibition of RT respectively. Each sample was tested three times for the reproducibility of its activity if present. The procedure followed is a modification of cDNA synthesis kit procured from Amersham International Ltd. Birminghamshire, UK.

ISOTOPIC ENZYME ASSAYS

Standard Isotopic RT-Inhibition Assay

Principle: as described in the electrophoretic RT-inhibition assay. The inhibitory activity is identified by using an isotope, tritiated thymidine triphosphate. A 50% or more reduction in the radioisotope uptake between the control and the test is taken as a positive inhibitory activity.

Procedure: The procedure followed was as described by Ono et al. (1989). The assay was performed in a reaction mixture that contained the following in a final volume of 50 μ l. Tris HCl 50 mM, P(rA)(dT)₁₂₋₁₈ 10 μ l/ml, BSA 10 μ g/ml, ³H-dTTP 0.5 mM, DTT 10 mM, MgCl₂ 3mM, MMLV RT 1 unit.

In the test, a known concentration of the formulation of the present invention prepared by the process described in the Examples 1 to 8 was added to the reaction mixture and incubated. Similarly a positive control (0.1 μ g/ml AZT), a negative control (distilled water) and a solvent control (solvent used in extract) were set up. Each set of test and controls were run in triplicate. After 30 minutes the reaction was stopped by adding 10 μ l of Ice cold EDTA (0.2M) and immersing the mixture in ice immediately.

Processing for radioactivity measurement:

After termination of the reaction, the DNA was precipitated using 10 μ l of cold 5% TCA and 0.1 M sodium pyrophosphate. 50 μ l of the reaction mixture was then filtered through Whatman DE81 filter paper. The filter paper was later washed thrice in 3 ml of 5% TCA and three times in absolute alcohol. The filters were then air dried, and radioactivity measured using a toluene-based scintillation cocktail. A reduction of 50 % or more in the radioactive count of the test from the negative control is taken as presence of RT inhibition activity. The results are presented in Table 5.

D. ANTI HEPATOTOXIC POTENTIALS OF THE PHARMACEUTICAL FORMULATION OF THE PRESENT INVENTION

Anti hepatotoxic potentials of formulation of the present invention prepared by the process described in the Examples 1 to 8 was assessed after challenging isolated rat hepatocytes with a known hepatotoxic compound β -galactosamine.

Isolation of rat hepatocytes

An adult rat of known weight preferably more than 125 gm was anaesthetized with ether and a midline incision were made. The liver was perfused through the portal vein with 30-50 ml of cold sodium citrate (0.027M) in calcium free locke's solution. During the perfusion, the liver gets blanched and fully distended. Perfusion is generally completed within 5 minutes of anaesthetizing the animal. The perfused liver is excised and washed well with the perfusion fluid and pressed in folds of sterile filter paper, weighed and cut into several small pieces and with a pair of scissors. A known wet weight of the liver (usually between 2-6 gm) was transferred with 5 volume of cold 0.25M sucrose into a sterile glass homogenizer and finely grounded. The cell suspension was filtered once, without application of pressure through 200-mesh brass gauze to remove strands of connective tissue and clumps of cells. The suspension contained some cell debris and blood cells at this stage, which were removed by centrifugation at a low speed (100-200g) for 2 minutes. After removal of the supernatant, the cell sediment was resuspended in a known volume of the minimum essential medium for further studies.

Cell count

100 μ l of the cells are taken and to this 300 μ l of 4% trypan blue is added and this is mixed and then viewed in the haemocytometer within 5 minutes of staining. The cells, which have not taken the dye, are live cells, which are counted.

The cells were inoculated into the culture medium which is composed of Eagle's MEM supplemented with 10% heat inactivated calf serum penicillin (100IU/ml), streptomycin (100IU/ml), 10^{-6} M Dexamethosone and 10^{-8} units Insulin. Inocula of 5×10^4 cells /0.1ml/cm³ were seeded into plastic dishes and preincubated in a humidified incubator at 37°C under 5% CO₂ in air for 24 hours and medium is replaced.

Study design

Four sets of isolated rat hepatocyte cultures were put up. Set I acted as control, Set II was treated with 1 mg/ml concentration of the formulation of the present invention prepared by the process described in the Examples 1 to 8. Set III with 0.5 mM concentration of β -galactosamine, a known hepatotoxic agent and set IV was treated with β -galactosamine and then protected with the formulation of the present invention. Culture supernatants of all the sets were assayed for glutamic pyruvate transaminase levels as per standard procedures.

Results

1. The study revealed that the formulation of the present invention by itself did not bring about any hepatotoxicity on rat hepatocytes,
2. β -galactosamine was proved as a profound hepatotoxic chemical.
3. The formulation of the present invention at 1mg/ml concentration was shown to protect isolated rat hepatocytes significantly from β -galactosamine induced hepatotoxicity.
4. Thus the study has proved that the formulation of the present invention has significant antihepatotoxic potentials ($p < 0.01$).

The results of the assay by the different formulations of the present invention prepared by the process described in the Examples 1 to 8 are given in Table 5.

E. INVITRO INHIBITION OF HBSAG SECRETION BY THE PHARMACEUTICAL FORMULATION OF THE PRESENT INVENTION

Alexander cell line (345) was kindly provided by Dr. Tim Harrison, Academic School of Medicine, Royal Free Hospital, London which is a continuous cell line of human hepatic cellular carcinoma cells (PLC/PRF/5). The cell line was cultured from a cancer patient who was also an HBsAg carrier. These cells grown *in vitro* secrete only HBsAg without any infectious virus.

Cultivation of Alexander cell line

Alexander cell line was grown as per the procedure adopted for the cultivation of Vero cell line described in section 4.6.2.2. 10% foetal calf serum (Sigma Chemical Company, USA) was used instead of 5% inactivated goat serum.

Study design

Six sets of Alexander cell line were grown in Leighton tubes. On day 1 of the experiment, the culture medium was decanted, and fresh medium was added. 1mg/ml concentration of formulation of the present invention prepared by the process described in the Examples 1 to 8 was added to each tube. The culture medium (supernatant) was assayed daily at varying doubling dilutions starting from neat to 1/128 dilution to check for the inhibition of secretion of HBsAg by the cell line. Distilled water was added to the control tubes. HBsAg detection from the supernatant was done using Hepanostika HBsAg kits as per the procedure described earlier.

Results

Inhibition of HBsAg secretion was observed for 48 hours when the cell line was treated with 1mg/ml concentration of the formulation as a single dose. However HBsAg was detected from the culture medium at lower dilutions after 72 hours. The details of the results of the formulations of the present invention prepared by the process described in the examples 1 to 10 are given in Table 5.

F. STUDY OF IMMUNOMODULATORY POTENTIALS OF THE PHARMACEUTICAL FORMULATION OF THE PRESENT INVENTION

- (a) The isolation of lymphocytes was done by ficoll-paque method using O group Rh+ve human blood. After centrifugation of the blood sample layered onto the Ficoll-paque at 400g for 20min at 18-20°C in a refrigerated centrifuge, lymphocytes are separated and suspended gently in 6-8 ml of balanced salt solution. It was centrifuged at 100g for 10 min at 18-20°C. After removing the supernatant, the lymphocytes are suspended in RPMI medium.

(b) Lymphocyte viability test: A cell suspension containing 5×10^6 cells/ml was prepared in RPMI medium. 0.5ml of 0.4% trypan blue solution was transferred to a test tube. To this 0.3ml of RPMI medium and 0.2ml of cell suspension were added and mixed thoroughly. The mixture was allowed to stand for 5 minutes. The suspension was viewed through a haemocytometer and looked for viable cells. The viable cells do not take up the dye.

% Viability was calculated by the formula =

$$\frac{\text{total viable cells (unstained)}}{\text{total cells (stained \& unstained)}} \times 100$$

(c) T-cell proliferation assay: To test by in-vitro method the T-cell proliferation inhibition/acceleration potentials of formulation of the present invention prepared by the process described in the Examples 1 to 8, which may be indicative of immunomodulatory potentials.

Requirements for the assay:

1. Peripheral blood lymphocytes (PBL)
2. Phytohaemagglutinin (PHA)
3. MTT (3,4,5-dimethyl thyl-2-yl-di phenyl tetrazolium bromide)
4. Acid propanol (0.40M HCl in isopropanol)
5. RPMI 1640 medium
6. Fetal calf serum
7. Antibiotics Vancomycin -25µg/ml
 Gentamycin-20µg/ml

Procedure of the assay:

1. Peripheral blood lymphocytes (PBL) were obtained by the Ficoll-hypaque method
2. Under sterile condition 50µl of PBL suspension (5×10^6 cells/ml), 50µl of sample dilutions and 50µl of PHA (33µg/ml) were added in the 96-well flat bottomed microtitre plate
3. Incubate the plates at 37°C and 5% CO₂ for 48 hrs
4. After incubation cell growth was quantified by adding 25µl of MTT to each well
5. Incubate the plates at 37°C for 4 hrs

6. 50µl of acid propanol was added and the content of each well was mixed thoroughly
7. Plates were read on automatic ELISA reader at 550nm.

Controls & tests in duplicate:

1. PBL+ PHA —→ 100% activity
2. PBL+ RPMI —→ 0% activity
3. PBL+ Picroliv —→ known Positive control
4. PBL+ formulations —→ Test samples
of the present invention

RESULTS:

The extracts were assessed for the lymphocyte proliferation activity by

- a) Microscopic: Multiwell plates were observed under inverted phase contrast microscope for any observable induction of proliferation. A minimum of 10 fields was observed.
- b) MTT calorimetric assay: The multiwell plates were incubated with MTT for 4 hrs. Then the plates were centrifuged at 100 rpm for 10 min. The supernatant was aspirated, 50µl of acid propanol was added and the content of each well was mixed thoroughly. The plates were read in an automated ELISA reader at 550nm. The proliferation of the lymphocytes was assessed by comparing with known immunomodulatory control (Picroliv).

The details of the results are given in Table 5.

Table 5: ANTIVIRAL AND BIOLOGICAL EFFICACY OF THE FORMULATIONS OBTAINED BY THE PROCESS DESCRIBED IN EXAMPLES 1 TO 10

EXAMPLES	ANTIVIRAL & BIOLOGICAL PROPERTIES (MIC /ml concentration)					
	HBsAg binding	HBV-DNAp inhibition	RT-inhibition	Inhibition of HBsAg secretion	Ant-hepatotoxicity	Immunomodulation
Example-1	2.5 mgm	400 µg	200 µg	1 mgm	1 mgm	400 µg
Example-2	1.25 mgm	100 µg	100 µg	0.5 mgm	1 mgm	100 µg
Example-3	2.5 mgm	400 µg	200 µg	1 mgm	1 mgm	400 µg
Example-4	1.25 mgm	200 µg	100 µg	1 mgm	1 mgm	200 µg
Example-5	5.0 mgm	400 µg	400 µg	2 mgm	1 mgm	400 µg
Example-6	2.5 mgm	200 µg	100 µg	1 mgm	1 mgm	200 µg
Example-7	2.5 mgm	400 µg	200 µg	2 mgm	1 mgm	400 µg
Example-8	1.25 mgm	200 µg	100 µg	1 mgm	1 mgm	200 µg
Example-9	2.5 mgm	400 µg	400 µg	2 mgm	-	-
Example-10	5.0 mgm	-	-	-	1 mgm	400 µg

Table 6 summarises the case studies on the efficacy of the new formulation of the present invention prepared by the process described in Example 1 for the treatment of acute and chronic Hepatitis C infections conducted at Chennai, India by Thyagarajan et al (1996-99) using the formulation

Table 6. Case studies on the efficacy of the new formulation of P. amarus for the treatment of acute and chronic Hepatitis C infections conducted at Chennai, India using the formulation obtained by the process described in Example 1 (Thyagarajan et al, unpublished)

Sl. No	Pt. Identity	Age/ Sex	Diagnosis & History	HCV		Pre-treatment parameters					P. amarus treatment period (months)	HCV		Pre-treatment parameters					S. bilirubin mgm %
				Ab	RNA	SGOT IU	SGPT IU	SAP IU	S. bilirubin Mgm %	Ab		RNA	SGOT IU	SGPT IU	SAP IU	S. bilirubin mgm %			
1	Mrs.M	45/F	PTH B&C After cardiac surgery	+	+	210	322	78	5.6		2m	+	-	25	34.2	44	1.2		
2	Mrs. U.B	42/F	PTH-C CPH after caesarian	+	ND	200	146	80	0.6		9m	+	ND	69	72	22	0.6		
3	Mr. V.R	52/ M	PTH-C CLD Cardiac surgery	+	ND	237	290	34	14.3		3m	+	ND	45	62	12	2.8		
4	Mrs. L.M	38/F	PTH-C AVH	+	+	562	360	34	0.9		3m	+	-	62	48	18	0.8		
5	Mrs. V.O	44/F	PTH-B&C AVH	+	+	111	173	44	0.9		4m	+	-	55	42	20	0.8		
6	Mrs. J.A	61/F	PTH-C AVH	+	+	162	213	123	0.9		3m	+	+	112	146	84	0.9		
7	Mrs. R.K	38/F	CAH Surgery + Transfusion+	+	+	404	581	88	1.5		6m	+	+	340	491	52	0.5		
8	Mr. R.I.R	32/ M	PTH-C	+	+	52	95	201	0.6		3m	+	+	42	38	62	0.5		
9	Mr.G.N	37/ M	Cirrhosis (B&C) Transfusion + Surgery+ Jaundice+	+	ND	60	100	444	13		6m	+	ND	56	72	120	5.2		

10	Mr.B.M	24/ M	Recurrent VH	+	-	69	108	ND	3.1	9m	+	-	35	24	-	1.0
11	Mrs. M.K	29/ M	PTH-AVH-C Surgery +	+	+	250	90	ND	5.7	5m	+	+	46	42	ND	1.8
12	Mr. SMA W	45/ M	CLD	+	+	76	47	127	1.9	6m	+	+	72	44	86	1.9
13	Mr.R	25/ M	PTH-AVH-C Blood trans+	+	ND	79	111	-	2.4	3m	+	ND	42	35	-	0.9
14	Mr. K.P.J	63/ M	PTH-AVH-C Surgery + Blood trans+	+	ND	207	233	280	1.6	3m	+	ND	48	42	51	0.9
15	Mr.S.V	29/ M	CRF: Dialysis	+	+	185	509	376	0.7	3m	+	+	61	85	78	0.7
16	Mr. M.C	55/ M	CAH	+	+	27	125	139	1.2	6m	+	+	20	54	115	0.9
17	Dr. S.K	29/ M	Asymptomatic c C ? occupational	+	+	52	66	72	0.6	6m	+	+	38	55	42	0.56
18	Mrs. V. R	50/F	PTH-AVH-C Blood trans+	+	ND	482	809	62	18.6	3m	+	ND	83	128	40	4.0

Inference: Total cases studied :18; Normalisation of enzymes :14/18 (77.8%); HCV-RNA negativity: 4/12 (33.3%) (By Qualitative PCR only) ; Physical well being and clinical improvement 17/18 (94.4%); Observable side effects : nil [Dosage: 500 mgms oral capsules of P.amarus-formulation (Example 1 x thrice daily x peroids mentioned against each case)]

In summary, the above findings confirm that when parts of medicinal plant, *Phyllanthus amarus* are extracted separately with a polar solvent alone, polar solvent and water in specific ratios and when such extracts are mixed together, the resultant formulation has all the essential antiviral and biological properties while the individual polar or aqueous extracts alone does not possess one or more of these properties.

It is our finding that if all the above said essential properties are made available in a single formulation, the resulting formulation will have uniform and stable antiviral and biological properties

G. BIOLOGICAL AND CHEMICAL STANDARDISATION OF THE PHARMACEUTICAL FORMULATION OF THE PRESENT INVENTION

As the antiviral activity of *P. amarus* was found to differ between collections made from different areas, the formulation preparation to be used in the clinical trial was standardized by evaluating their antiviral properties and matching them using the HPLC pattern upon fractionation, initially and later upon the preparation of the pharmaceutical formulations of the present invention prepared by the process mentioned in Examples 1 to 8.

Collection of plant material : *Phyllanthus amarus* was collected from different places within Chennai city and within Tamilnadu and Bangalore. They were Salem, Mathuranthagam, Coimbatore, Tiruchy, Tiruttani, Bangalore from outside Madras; Annanagar, Thiruvannamiyur and Tiruvattiyur from in and around Madras. All these plant materials were identified based on the taxonomical system of nomenclature.

Preparation of the formulation for HPLC:

The formulations of the present invention prepared by the process explained in Examples 1 to 8 from various collections as described earlier under the head "embodiments". The formulations so obtained were filtered and made upto 50ml. 10ml of each extract was used as such for chemical standardisation study. The rest was dried and used for antiviral and biological testing by a battery of tests as described in A to F. Chemical fingerprinting was performed using a High performance liquid chromatogram for each of the extract and formulation.

HPLC analysis:**Requirements :**

1. HPLC grade methanol
2. HPLC grade water
3. Shimpack PrepODS(K) kit (4.6 mmid x 25 cm, particle dia 5 μ m Rreverse phase column)
(procured from Shimadzu Asia Pacific PTE ltd., Singapore)

Equipment required:

HPLC- A Shimadzu chromatographic system comprising

1. LC-6A liquid pump
2. SCL-6B system controller
3. Model 7125 injector
4. SPD-6A UV detector

Chromatographic system:

Column	:	Shimpack PrepODS (K) kit (4.6 mmid x 25 cm, particle dia 5- μ m Reverse phase column)
Mobile phase	:	Methanol: water gradient
Flow rate	:	2ml/min
Detection	:	UV 225 nm
Injection volume	:	10 μ l

Procedure: The formulations of the present invention prepared for the study was filtered through 0.2 μ membrane filter discs. The filtrate was used for the study. After the column was washed, to achieve a baseline, the gradient parameters were set and the run was started by injecting 10 μ l of the formulation. The chromatogram was plotted. Similarly chromatographic patterns were plotted for all the formulations. The chromatograms got were compared and analysed for their matching antiviral and biological properties as described in A to F. The formulations prepared as per the process explained in Examples 1 to 8 were found to have all

the above mentioned properties optimally as shown in Table 6 and to possess the HPLC pattern depicted in Fig 1 to 10 of the drawings accompanying this specification which represents the HPLC pattern of the formulation prepared by the process described in Example 1 to 10.

The pharmaceutical formulation of the present invention can be administered to a person who requires such administration by the normal means such as tablets , capsules , oral suspensions and the like. The dosage of administration of the formulation may range from 250 mg to 500 mg thrice daily for a duration of 1 to 6 months time depending upon the clinical conditions of the person who requires the administration of the formulation

Advantages of the invention

The pharmaceutical formulation of the present invention can find applications for the treatment of acute and chronic Hepatitis B; acute and chronic Hepatitis C and other related viral infections of the liver because

- 1. The formulation has all the essential antiviral and biological properties against Hepatitis B virus; Hepatitis C virus and other related viral infections of the liver.**
- 2. The formulation is non-toxic at tissue and cellular level; with proven safety for human use by experimental studies and clinical trials.**
- 3. The formulation possess reproducible clinical efficacy in the treatment of acute and chronic Hepatitis B; acute and chronic Hepatitis C and other related viral infections of the liver.**

Claims

- 1.** A pharmaceutical formulation useful for the treatment of Hepatitis B (both acute and chronic), Hepatitis C (both acute and chronic) and other related viral infections of the liver with antihepatotoxic and liver cell regenerating potentials and immunomodulating properties which comprises
 - (i) an extract of the plant *Phyllanthus amarus* obtained by using a polar solvent alone
 - (ii) an extract of the plant *Phyllanthus amarus* obtained by using a mixture of a polar solvent and water and
 - (iii) an extract of the plant *Phyllanthus amarus* obtained by using water alone
- 2.** A pharmaceutical formulation as claimed in claim 1 which comprises
 - (i) 10 to 40% w/w of an extract of the plant *Phyllanthus amarus* obtained by using a polar solvent alone
 - (ii) 10 to 40% w/w of an extract of the plant *Phyllanthus amarus* obtained by using a mixture of a polar solvent and water wherein the ratio of the solvent to water ranges from 20 to 80 and 80 to 20% w/w respectively and
 - (iii) 10 to 40% w/w of an extract of the plant *Phyllanthus amarus* obtained by using water alone
- 3.** A pharmaceutical formulation as claimed in claims 1 & 2 which comprises
 - (i) 20 to 30% w/w of an extract of the plant *Phyllanthus amarus* obtained by using a polar solvent alone
 - (ii) 20 to 40% w/w of an extract of the plant *Phyllanthus amarus* obtained by using a mixture of a polar solvent and water wherein the ratio of the solvent to water ranges from 80 to 20% and 20 to 80% w/w respectively
 - (iii) 20 to 40% w/w of an extract of the plant *Phyllanthus amarus* obtained by using a polar solvent and water wherein the solvent to water ratio ranges from 20 to 80 % and 80 to 20 % w/w respectively and
 - (iv) 20 to 30% w/w of an extract of the plant *Phyllanthus amarus* obtained by using water alone

4. A pharmaceutical formulation as claimed in Claims 1 to 3 which comprises **one part each of**
- (i) an extract of the plant *Phyllanthus amarus* obtained by using a polar solvent alone ,
 - (ii) an extract of the plant *Phyllanthus amarus* obtained by using a mixture of a polar solvent and water and
 - (iii) an extract of the plant *Phyllanthus amarus* using water alone
5. A pharmaceutical formulation as claimed in claims 1 to 4 which comprises **one part each of**
- (i) 10 to 40% w/w of an extract of the plant *Phyllanthus amarus* obtained by using a polar solvent alone
 - (ii) 10 to 40% w/w of an extract of the plant *Phyllanthus amarus* obtained by using a mixture of a polar solvent and water wherein the ratio of the solvent to water ranges from 20 to 80 and 80 to 20% w/w respectively and
 - (iii) 10 to 40% w/w of an extract of the plant *Phyllanthus amarus* obtained by using water alone
6. A pharmaceutical formulation as claimed in claims 1 to 5 which comprises **one part each of**
- (i) 20 to 30 %w/w of an extract of the plant *Phyllanthus amarus* obtained by using a polar solvent alone ,
 - (ii) 20 to 40 %w/w of an extract of the plant *Phyllanthus amarus* obtained by using a mixture of a polar solvent and water wherein the ratio of the solvent to water ranges from 80 to 20% and 20 to 80 % w/w respectively
 - (iii) 20 to 40 % w/w of an extract of the plant *Phyllanthus amarus* using a mixture of a polar solvent and water wherein the ratio of the solvent to water ranges from 20 to 80 % w/w and 80 to 20 % w/w respectively and
 - (iv) 20 to 30 % w/w of an extract obtained using water alone

7. A pharmaceutical formulation as claims in claims 1 to 6 which comprises two parts of

- (i) of an extract of the plant *Phyllanthus amarus* obtained by using a polar solvent alone
an extract of the plant *Phyllanthus amarus* obtained by using a polar solvent and **one part each of**
- (ii) of an extract of the plant *Phyllanthus amarus* obtained by using a mixture of a polar solvent and water and
- (iii) an extract of the plant *Phyllanthus amarus* using water alone

8. A pharmaceutical formulation as claimed in claims 1 to 7 which comprises two parts

- (i) of 10 to 40 %w/w of an extract of the plant *Phyllanthus amarus* obtained by using a polar solvent **and one part each**
- (ii) of 10 to 40 % w/w of an extract of the plant *Phyllanthus amarus* obtained by using a mixture of a polar solvent and water wherein the ratio of the solvent to water ranges from 20 to 80% and 80 to 20 % w/w respectively
- (iii) of 10 to 40% w/w of an extract of the plant *Phyllanthus amarus* obtained by using water alone

9. A pharmaceutical formulation as claimed in claims 1 to 8 which comprises two parts

- (i) of 20 to 30 % W/W of an extract of the plant *Phyllanthus amarus* obtained by using a polar solvent alone **and one part each**
- (ii) of 20 to 40 % w/w of an extract of the plant *Phyllanthus amarus* obtained by using a mixture of a polar solvent and water wherein the ratio of the solvent to water ranges from 80 to 20% and 20 to 80 % w/w respectively
- (iii) of 20 to 40 % w/w of an extract of the plant *Phyllanthus amarus* obtained by using a mixture of polar solvent and water wherein the ratio of the solvent to water ranges from 20 to 80% w/w and 80 to 20% w/w respectively and
- (iv) of 20 to 30 % w/w of an extract of the plant *Phyllanthus amarus* obtained by using water alone.

10.A process for the preparation of pharmaceutical formulation useful for the treatment of Hepatitis B (acute and chronic), Hepatitis C (both acute and chronic) and other related viral infections of the liver with antihepatotoxic and liver cell regenerating potentials and immunomodulating properties which comprises mixing

- (i) an extract of the plant *Phyllanthus amarus* obtained by using a polar solvent alone
- (ii) an extract of the plant *Phyllanthus amarus* obtained by using a mixture of a polar solvent and water and
- (iii) an extract of the plant *Phyllanthus amarus* obtained by using water alone

11.A process as claimed in claim in 10 which comprises mixing

- (i) 10 to 40% w/w of an extract of the plant *Phyllanthus amarus* obtained by using a polar solvent alone
- (ii) 10 to 40% w/w of an extract of the plant *Phyllanthus amarus* obtained by using a mixture of a polar solvent and water wherein the ratio of the solvent to water ranges from 20 to 80 and 80 to 20% w/w respectively and
- (iii) 10 to 40% w/w of an extract of the plant *Phyllanthus amarus* obtained by using water alone

12.A process as claimed in claims 10 & 11 which comprises mixing

- (i) 20 to 30% w/w of an extract of the plant *Phyllanthus amarus* obtained by using a polar solvent alone
- (ii) 20 to 40% w/w of an extract of the plant *Phyllanthus amarus* obtained by using a mixture of a polar solvent and water wherein the ratio of the solvent to water ranges from 80 to 20% and 20 to 80% w/w respectively
- (iii) 20 to 40-% w/w of an extract of the plant *Phyllanthus amarus* obtained by using a polar solvent and water wherein the solvent to water ratio ranges from 20 to 80 % and 80 to 20 % w/w respectively and
- (iv) 20 to 30% w/w of an extract of the plant *Phyllanthus amarus* obtained by using water alone

13.A process as claimed in claim 10 to 12 which comprises mixing **one part each of**

- (i) an extract of the plant *Phyllanthus amarus* obtained by using a polar solvent alone ,
- (ii) an extract of the plant *Phyllanthus amarus* obtained by using a mixture of a polar solvent and water and
- (iii) an extract of the plant *Phyllanthus amarus* using water alone

14.A process as claimed in claims 10 to 13 which comprises mixing **one part each of**

- (i) 10 to 40% w/w of an extract of the plant *Phyllanthus amarus* obtained by using a polar solvent alone
- (ii) 10 to 40% w/w of an extract of the plant *Phyllanthus amarus* obtained by using a mixture of a polar solvent and water wherein the ratio of the solvent to water ranges from 20 to 80 and 80 to 20% w/w respectively and
- (iii) 10 to 40% w/w of an extract of the plant *Phyllanthus amarus* obtained by using water alone

15.A process as claimed in claims 10 to 14 which comprises mixing **one part each of**

- (i) 20 to 30 %w/w of an extract of the plant *Phyllanthus amarus* obtained by using a polar solvent alone ,
- (ii) 20 to 40 %w/w of an extract of the plant *Phyllanthus amarus* obtained by using a mixture of a polar solvent and water wherein the ratio of the solvent to water ranges from 80 to 20% and 20 to 80 % w/w respectively
- (iii) 20 to 40 % w/w of an extract of the plant *Phyllanthus amarus* using a mixture of a polar solvent and water wherein the ratio of the solvent to water ranges from 20 to 80 % w/w and 80 to 20 % w/w respectively and
- (iv) 20 to 30 % w/w of an extract obtained using water alone

16.A process as claimed in claims 10 to 15 which comprises mixing **two parts**

- (i) of an extract of the plant *Phyllanthus amarus* obtained by using a polar solvent alone **one part each of**

- (ii) of an extract of the plant *Phyllanthus amarus* obtained by using a mixture of a polar solvent and water and
- (iii) an extract of the plant *Phyllanthus amarus* using water alone

17. A process as claimed in claims 10 to 16 which comprises **mixing two parts**

- (i) of 10 to 40 %w/w of an extract of the plant *Phyllanthus amarus* obtained by using a polar solvent **and one part each**
- (ii) of 10 to 40 % w/w of an extract of the plant *Phyllanthus amarus* obtained by using a mixture of a polar solvent and water wherein the ratio of the solvent to water ranges from 20 to 80% and 80 to 20 % w/w respectively
- (iii) of 10 to 40% w/w of an extract of the plant *Phyllanthus amarus* obtained by using water alone

18. A process as claimed in claims 10 to 17 which comprises mixing **two parts**

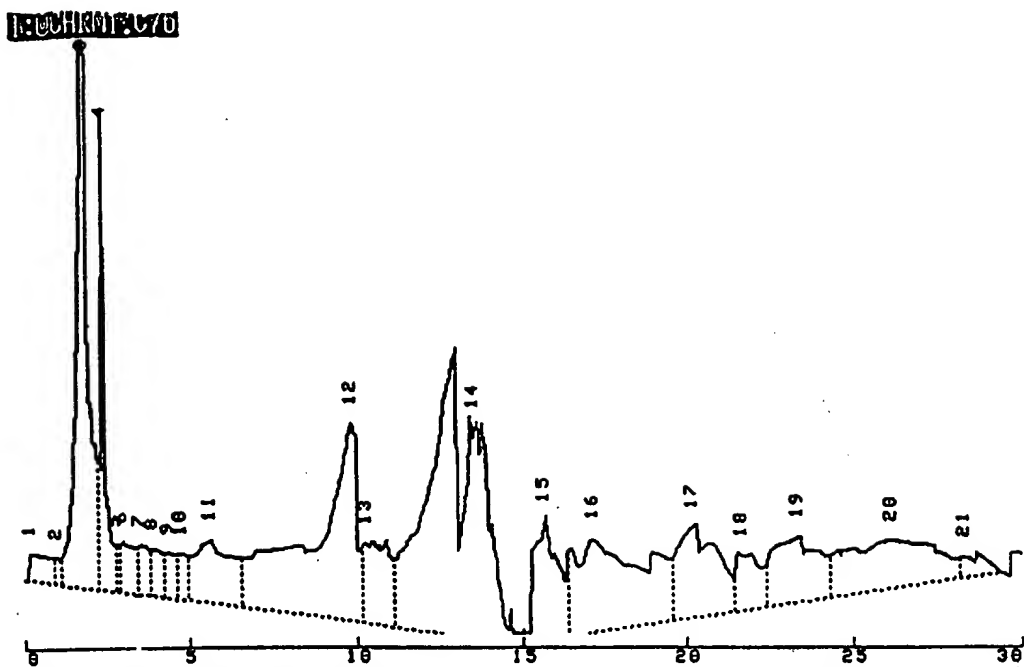
- (i) of 20 to 30 % W/W of an extract of the plant *Phyllanthus amarus* obtained by using a polar solvent alone **and one part each**
- (ii) of 20 to 40 % w/w of an extract of the plant *Phyllanthus amarus* obtained by using a mixture of a polar solvent and water wherein the ratio of the solvent to water ranges from 80 to 20% and 20 to 80 % w/w respectively
- (iii) of 20 to 40 % w/w of an extract of the plant *Phyllanthus amarus* obtained by using a mixture of polar solvent and water wherein the ratio of the solvent to water ranges from 20 to 80% w/w and 80 to 20% w/w respectively and
- (iv) of 20 to 30 % w/w of an extract of the plant *Phyllanthus amarus* obtained by using water alone.

19. A process as claimed in claims 10 to 18 wherein the extracts are prepared from the powder obtained by drying the parts such as leaves, stems, seeds and roots of the taxonomically identified collections of the plant *Phyllanthus amarus*, keeping it in an oven heated to a temperature in the range of 50 to 80 degree C for a period in the range of 3 to 5 hrs a day for a period of 3 to 6 successive days and powdering

- 20.**A process as claimed in claim 19 wherein before subjecting the powder for extraction procedures, each batch of powder is subjected for known sterilising treatment , if required for removing any bacterial or fungal contamination
- 21.**A process as claimed in claims 10 to 20 wherein the extraction is carried out at a temperature in the range of 37 to 60 degree C for a period ranging from 2 hours to 18 hours
- 22.**A process as claimed in claims 10 to 21 wherein the polar solvent such as methanol, ethanol, hexane and butanol is used for the extraction .
- 23.**A pharmaceutical formulation useful for the treatment of Hepatitis B (both acute and chronic) ; Hepatitis C (both acute and chronic) and other related viral infections of the liver with antihepatotoxic and liver cell regenerating potentials and immunomodulating properties substantially as herein described with reference to the Examples 1 to 8.
- 24.**A process for the preparation of a pharmaceutical formulation useful for the treatment of Hepatitis B (both acute and chronic); Hepatitis C (both acute and chronic) and other related viral infections of the liver with antihepatotoxic and liver cell regenerating potentials and immunomodulating properties substantially as herein described with reference to the Examples 1 to 8.
- 25.**Use of the pharmaceutical composition as claimed in claims 1 to 9 for the preparation of a medicament useful for treating Hepatitis B (both acute and chronic), Hepatitis C (both acute and chronic) and other related viral infections of the liver.
- 26.**Use of the pharmaceutical composition as claimed in claims 1 to 9 in the treatment of Hepatitis B (both acute and chronic), Hepatitis C (both acute and chronic) and other related viral infections of the liver.
- 27.**Use of the pharmaceutical composition as claimed in claims 1 to 9 for achieving antihepatotoxicity, liver cell regeneration and immunomodulation.
- 28.** Use of the pharmaceutical composition as claimed in any of the claims 25 to 27 wherein the composition is prepared by the process as claimed in claims 11 to 22.

- 29.** A method of treating Hepatitis B (both acute and chronic), Hepatitis C (both acute and chronic) and other related viral infections of the liver which comprises administering a therapeutically effective dose of the pharmaceutical composition as claimed in claims 1 to 9.
- 30.** Method of claim 29 wherein the pharmaceutical composition is prepared by a process as claimed in claims 11 to 22.

1/10



MC MS SO PP MO CA PR E

<PC> Peak-top Comments

C-R4A CHROMATOPAC

CH=

REPORT No.=2

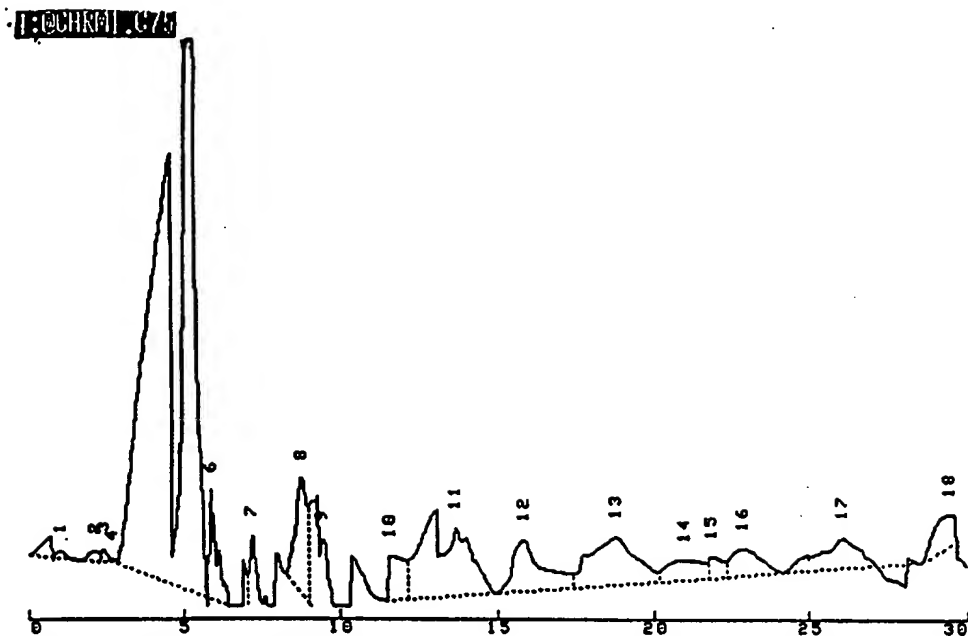
CHROMATOGRAM=1:@CHRM1.C76

99/02/15 10:00

** CALCULATION REPORT **

CH	PKNO	TIME	AREA	HEIGHT	MK	IDNO	CONC	NAME
1	1	0.167	18233	413			0.9329	
	2	1.018	4453	407	V		0.2279	
	3	1.877	260130	17023	V		13.3094	
	4	2.436	53756	6892	V		2.7504	
	5	2.825	4891	700	V		0.2502	
	6	2.995	22666	755	V		1.1597	
	7	3.528	16446	735	V		0.8414	
	8	3.878	18492	701	V		0.9461	
	9	4.353	14456	660	V		0.7396	
	10	4.738	12572	668	V		0.6432	
	11	5.601	71795	931	V		3.6733	
	12	9.837	271522	2879	V		13.8923	
	13	10.247	68388	1167	V		3.499	
	14	13.548	426941	3067	Y		21.8442	
	15	15.694	88106	1613	V		4.5079	
	16	17.174	201434	1354	V		10.3062	
	17	20.172	113040	1334	V		5.7836	
	18	21.689	40338	823	V		2.0639	
	19	23.325	90602	971	V		4.6356	
	20	25.116	139062	710	V		7.115	
	21	28.265	17164	314	V		0.8782	
TOTAL			1954486	44117			100	

Fig 1



MC MS SO PP MO CA PR E

<PC> Peak-top Comments

C-R4A CHROMATOPAC CH=1 REPORT No.=3 CHROMATOGRAM=1:CHRM1.C75 99.02.12 12:45:37

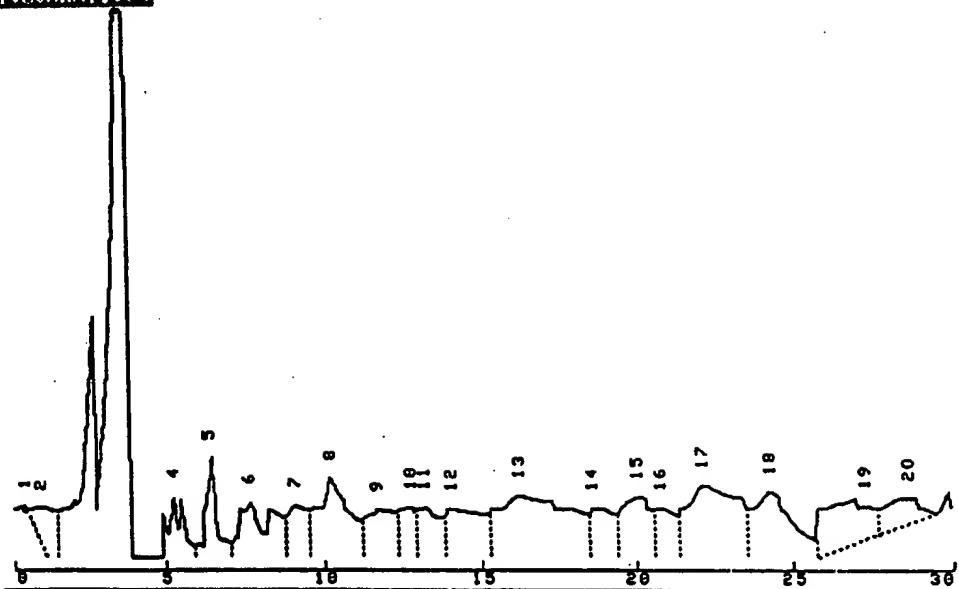
** CALCULATION REPORT **

CH	PKNO	TIME	AREA	HEIGHT	MK	IDNO	CONC	NAME
1	1	1.037	9990	113			0.8222	
	2	2.149	3372	144			0.2776	
	3	2.429	2383	162	V		0.1962	
	4	2.684	494	59			0.0407	
	5	5.239	663121	10971	V		54.5816	
	6	5.914	5598	1669			0.4607	
	7	7.212	24533	1237	V		2.0193	
	8	8.789	45556	1834			3.7497	
	9	9.476	64921	1324	V		5.3437	
	10	11.602	22766	697			1.8739	
	11	13.704	122955	1041	V		10.1204	
	12	15.859	53048	794	V		4.3664	
	13	18.823	75870	740	V		6.2449	
	14	20.957	24782	303	V		2.0398	
	15	21.839	10377	327	V		0.8541	
	16	22.855	28411	410	V		2.3385	
	17	26.058	32309	467	V		2.6593	
	18	29.442	24431	490			2.0109	
TOTAL			1214916	22781			100	

Fig 2

3/10

1:CHRM1.C64



MC MS SO PP MO CA PR E

<PC> Peak-top Comments

C-R4A CHROMATOPAC CH=1 REPORT No.=2 CHROMATOGRAM=1:CHRM1.C64 99/02 09 15:03:28

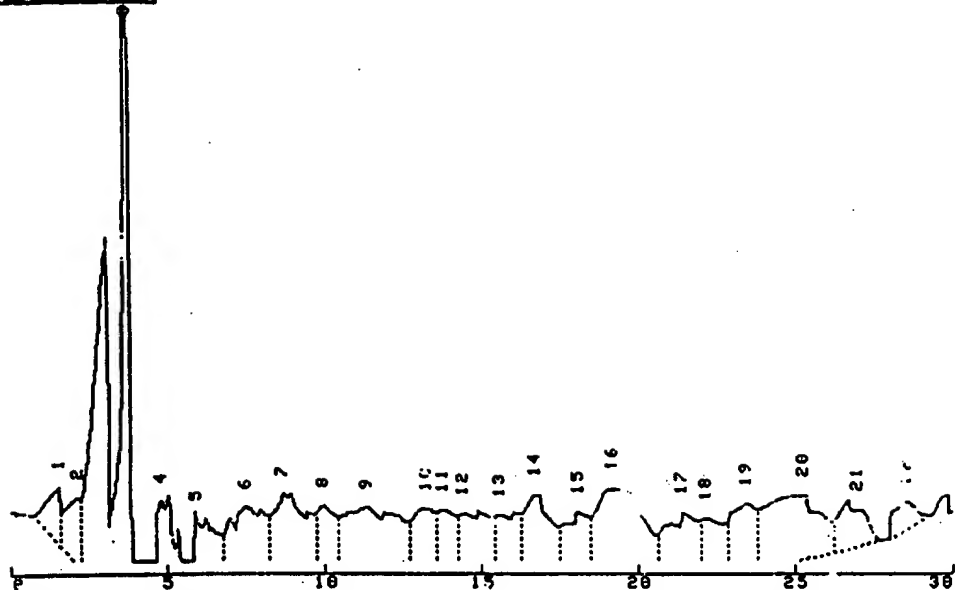
** CALCULATION REPORT **

CH	PKNO	TIME	AREA	HEIGHT	MK	IDNO	CONC	NAME
1	1	0.375	1113	90			0.0252	
	2	0.958	40336	720			0.9119	
	3	3.653	831417	18416	VE		18.7958	
	4	5.263	271922	4798	E		6.1473	
	5	6.425	287619	5234	V		6.5022	
	6	7.654	400784	4324	V		9.0605	
	7	9.075	188959	4009	V		4.2718	
	8	10.192	379882	4232	V		8.588	
	9	11.703	229913	3446	V		5.1976	
	10	12.775	118068	3264	V		2.6692	
	11	13.212	172084	3176	V		3.8903	
	12	14.037	240926	3012	V		5.4466	
	13	16.26	495904	2798	V		11.2109	
	14	18.642	106571	2134	V		2.4092	
	15	20.086	141461	2044	V		3.198	
	16	20.806	79389	1727	V		1.7947	
	17	22.177	206128	1831	V		4.6599	
	18	24.281	120736	1320	V		2.7295	
	19	27.28	75691	529	V		1.7111	
	20	28.631	34513	398	V		0.7802	
TOTAL			4423414	67502			100	

Fig 3

4/10

1:CHRM1.C62



MC MS SO PP MO CA PR E

<PG> Peak-top Comments

C-R4A CHROMATOPAC CH=1

REPORT No.=1

CHROMATOGRAM=1:CHRM1.C63

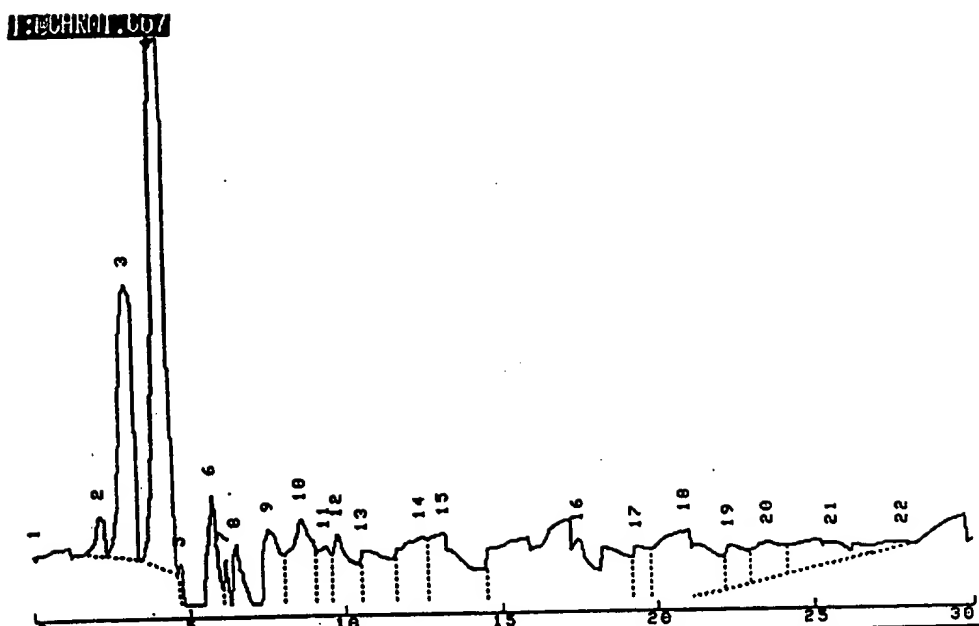
99-02-08 12:06:34

** CALCULATION REPORT **

CH	PKNO	TIME	AREA	HEIGHT	MK	IDNO	CONC	NAME
1	1	1.56	24552	914			0.838	
	2	2.15	31823	1081	V		1.0861	
	3	3.806	396657	12404	V		3.5382	
	4	4.839	164696	3318			5.6222	
	5	5.924	169940	2930	V		5.8092	
	6	7.466	244520	2921	V		8.3457	
	7	8.674	240990	2980	V		8.2252	
	8	9.957	112405	2658	V		3.8365	
	9	11.322	316776	2461	V		10.8118	
	10	13.275	113119	2218	V		3.8608	
	11	13.815	91360	2117	V		3.1182	
	12	14.46	130731	1988	V		4.462	
	13	15.524	93697	1831	V		3.1979	
	14	16.751	133126	2016	V		4.5437	
	15	18.127	83247	1577	V		2.8413	
	16	19.197	195454	1933	V		6.671	
	17	21.407	84345	1186	V		2.8788	
	18	22.139	48895	1011	V		1.6688	
	19	23.447	61796	1095	V		2.1091	
	20	25.276	127645	1015	V		4.3566	
	21	26.95	42554	584	V		1.4524	
	22	28.546	21583	424			0.7567	
TOTAL			2929908	50500			100	

Fig. 4

5/10



PC MC MS SO PP MO CA PR E

<PC> Peak-top Comments

C-R4A CHROMATOPAC CH=1 REPORT No.=2 CHROMATOGRAM=1:CHRM1.C67 99/02/16 15:33:43

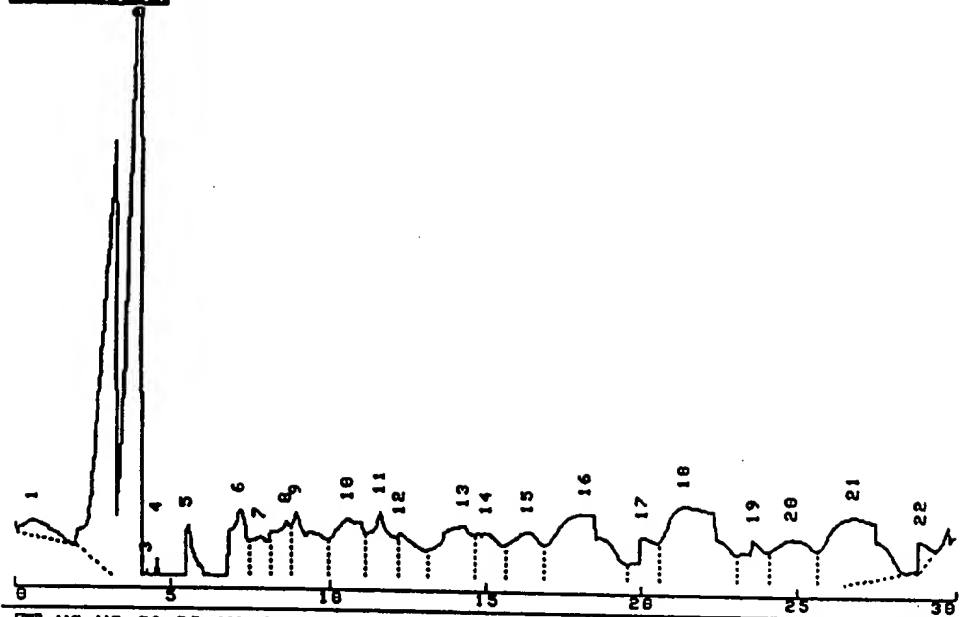
** CALCULATION REPORT **

CH	PKNO	TIME	AREA	HEIGHT	MK	IDNO	CONC	NAME
1	1	0.083	267	49			0.0119	
	2	2.183	13316	613			0.5941	
	3	3.139	132750	4265			5.9224	
	4	4.178	335522	13555			14.9687	
	5	4.734	7783	769			0.3472	
	6	5.784	86812	3464			3.873	
	7	6.159	36059	2382	V		1.6087	
	8	6.522	108520	2585	V		4.8414	
	9	7.556	107203	2697	V		4.7827	
	10	8.59	146833	2744	V		6.5507	
	11	9.336	69597	2222	V		3.1049	
	12	9.758	113737	2345	V		5.0742	
	13	10.585	128299	2010	V		5.7238	
	14	12.474	120696	1985	V		5.3846	
	15	13.192	178658	1957	V		7.9705	
	16	17.48	373297	1349	V		16.634	
	17	19.353	34633	1004	V		1.5451	
	18	20.938	122545	1067	V		5.4671	
	19	22.286	28797	672	V		1.2847	
	20	23.539	38284	571	V		1.708	
	21	25.565	50635	328	V		2.259	
	22	27.824	7247	63	V		0.3233	
TOTAL			2241488	48693			100	

Fig 5

6/10

1:CHRM1.C69



MC MS SO PP MO CA PR E

<PC> Peak-top Comments

C-R4A CHROMATOPAC

CH=1

REPORT No.=2

CHROMATOGRAM=1:CHRM1.C69

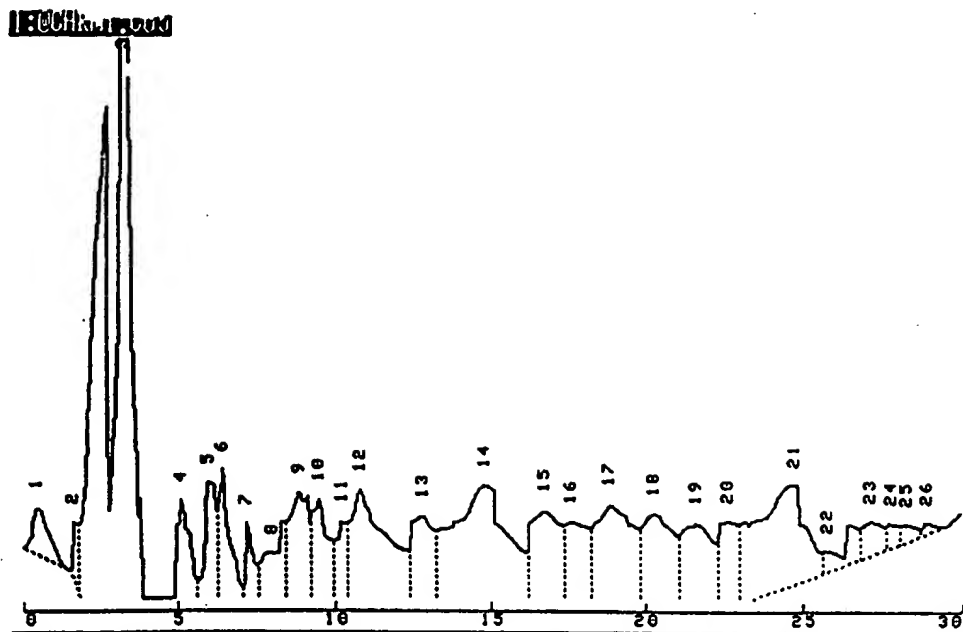
99 02 11 12:01:02

** CALCULATION REPORT **

CH	PKNO	TIME	AREA	HEIGHT	MK	IDNO	CONC	NAME
1	1	0.57	15572	236			0.5245	
	2	3.942	492061	13065			16.574	
	3	4.234	7342	756			0.2473	
	4	4.562	50510	1880			1.7013	
	5	5.562	142045	3086			4.7845	
	6	7.166	159866	3144	V		5.3847	
	7	7.789	95247	2677	V		3.2082	
	8	8.599	109771	2827	V		3.6974	
	9	8.916	183793	2928	V		6.1907	
	10	10.559	179376	2665	V		6.0419	
	11	11.589	158257	2659	V		5.3306	
	12	12.252	117273	2266	V		3.9501	
	13	14.257	191373	2183	V		6.446	
	14	15.014	114958	1993	V		3.8721	
	15	16.366	136277	1875	V		4.5902	
	16	18.217	266283	1950	V		8.9692	
	17	20.067	74176	1408	V		2.4984	
	18	21.4	220972	1747	V		7.443	
	19	23.627	51892	997	V		1.7479	
	20	24.849	75039	893	V		2.5275	
	21	26.758	118084	1023	V		3.9774	
	22	28.888	8700	402			0.2931	
TOTAL			2968867	52659			100	

Lg 6

7/10



PC MC MS SO PP MO CA PR E

<PC> Peak-top Comments

C-R4A CHROMATOPAC CH=1

REPORT No.=2

CHROMATOGRAM=1:CHRM1.C65

99:02 09 16:02:53

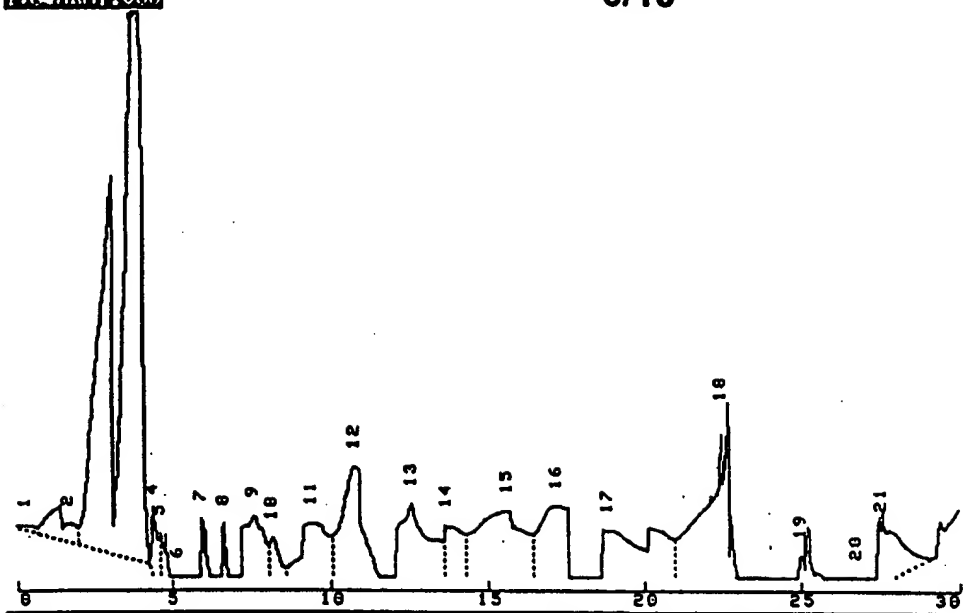
** CALCULATION REPORT **

CH	PKNO	TIME	AREA	HEIGHT	MK	IDNO	CONC	NAME
1	1	0.473	28591	739			0.6621	
	2	1.646	10020	958			0.232	
	3	3.317	806145	14547	VE		18.6681	
	4	5.136	179994	5030			4.1682	
	5	5.974	171127	5161	V		3.9628	
	6	6.4	210063	5268	V		4.8645	
	7	7.148	99411	4295	V		2.3021	
	8	7.975	194665	3679	V		4.5079	
	9	8.793	203648	4460	V		4.7159	
	10	9.457	178132	4229	V		4.125	
	11	10.192	93670	3730	V		2.1691	
	12	10.776	419825	4127	V		9.722	
	13	12.816	161070	3325	V		3.7299	
	14	14.823	521812	3424	V		12.0837	
	15	16.793	181695	2643	V		4.2075	
	16	17.66	116529	2316	V		2.6985	
	17	18.908	195783	2343	V		4.5338	
	18	20.263	134488	1938	V		3.1144	
	19	21.594	102152	1549	V		2.3655	
	20	22.644	59025	1375	V		1.3668	
	21	24.713	179567	1560	V		4.1583	
	22	25.808	26551	314	V		0.6148	
	23	27.144	22506	513	V		0.5212	
	24	27.756	8233	365	V		0.1906	
	25	28.275	9080	261	V		0.2103	
	26	28.875	4530	164	V		0.1049	
TOTAL			4318306	78314			100	

Fig 7

12-CHRM1.C36

8/10



MC MS SO PP MO CA PR E

<PC> Peak-top Comments

C-R4A CHROMATOPAC

CH=1

REPORT No.=2

CHROMATOGRAM=1:@CHRM1.C36

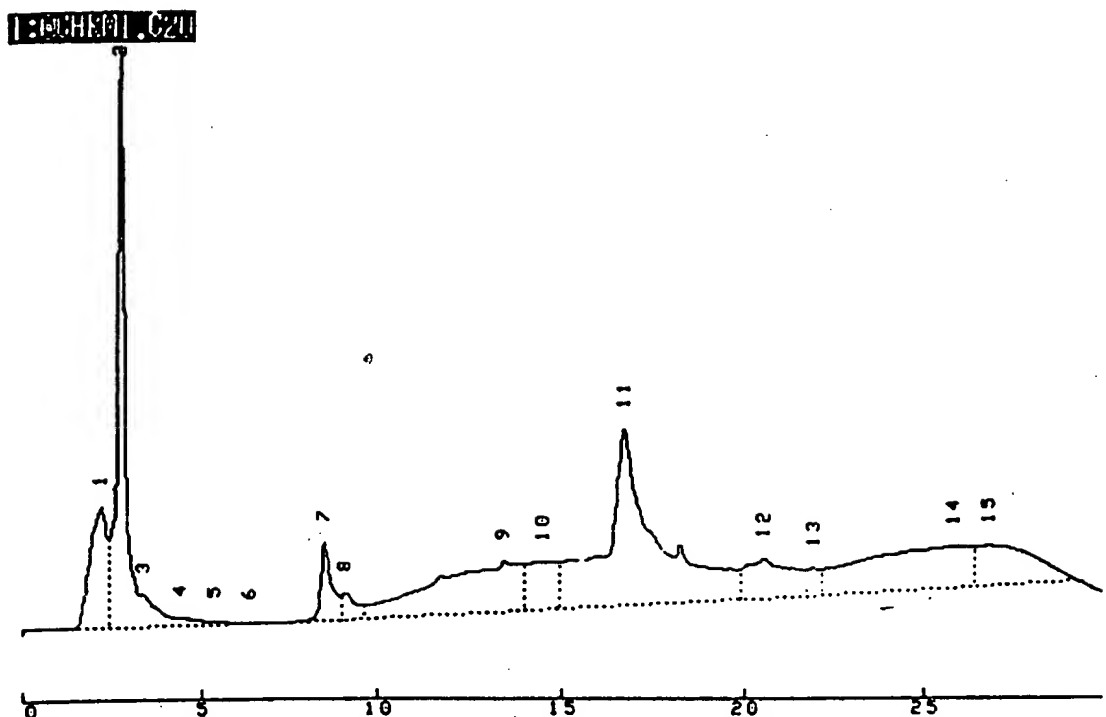
99/02/24 09:03:36

** CALCULATION REPORT **

CH	PKNO	TIME	AREA	HEIGHT	MK	IDNO	CONC	NAME
1	1	0.317	2933	92			0.0962	
	2	1.714	25970	299	V		0.8519	
	3	3.887	551431	13840	V		18.0889	
	4	4.407	16583	1091			0.544	
	5	4.634	24561	1199	V		0.8057	
	6	5.175	7792	969			0.2556	
	7	5.941	68791	3481			2.2566	
	8	6.597	128163	3311	V		4.2042	
	9	7.551	163905	3358	V		5.3767	
	10	8.135	86289	2946	V		2.8306	
	11	9.361	245548	3047	V		8.0548	
	12	10.752	309416	3796	V		10.1499	
	13	12.546	250642	2995	V		8.2219	
	14	13.698	102576	2518	V		3.3649	
	15	15.603	305161	2555	V		10.0104	
	16	17.237	192929	2458	V		6.3288	
	17	18.89	235216	1883	V		7.7159	
	18	22.408	234219	2916	V		7.6832	
	19	25.016	42344	914			1.389	
	20	26.7	5247	85	V		0.1721	
	21	27.521	48739	1052			1.5988	
TOTAL			3044453	54803			100	

Fig 8

9/10



MC MS SO PP MO CA PR E

<PC. Peak-top Comments

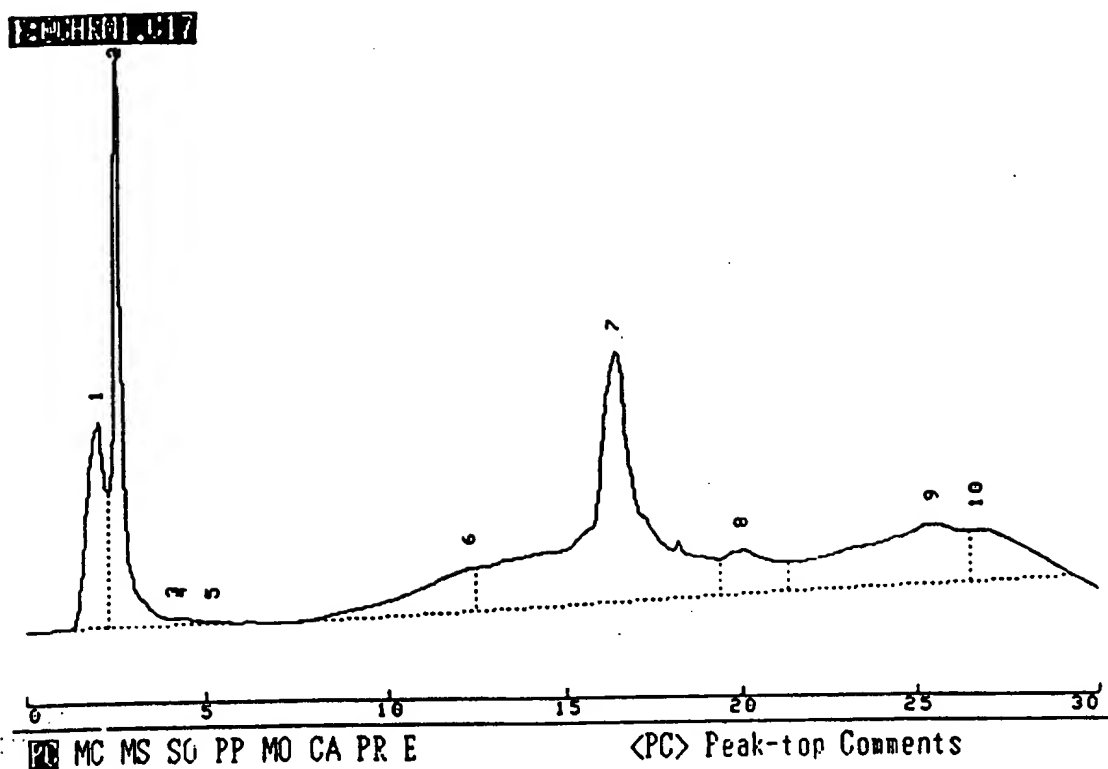
** CALCULATION REPORT **

97 03 29 10:11:55

CH	PKNO	TIME	AREA	HEIGHT	MK	IDNO	CONC	NAME
1	1	2.214	26238	823			6.1297	
	2	2.732	68384	4371	SV		15.9759	
	3	3.383	199	17	T		0.0466	
	4	4.433	145	6	T		0.0339	
	5	5.4	43	4	T		0.0101	
	6	6.371	81	5	T		0.019	
	7	8.476	11458	527			2.6768	
	8	9.103	5146	174	V		1.2021	
	9	13.431	56914	338	V		13.2962	
	10	14.533	17485	315	V		4.0847	
	11	16.724	118858	1198	V		27.7677	
	12	20.519	23182	268	V		5.4157	
	13	21.933	5004	182	V		1.169	
	14	25.8	61774	284	V		14.4316	
	15	26.794	33135	280	V		7.741	
TOTAL			428044	8792			100	

Fig. 9

10/10



** CALCULATION REPORT **

97.03 29 13:29:55

CH	PKNO	TIME	AREA	HEIGHT	MK	IDNO	CONC	NAME
1	1	2.075	46869	1391			8.983	
	2	2.587	78622	4474	SV		15.0687	
	3	4.169	39	5	T		0.0074	
	4	4.375	177	8	TV		0.0339	
	5	5.208	23	2	T		0.0045	
	6	12.308	37146	293			7.1194	
	7	16.445	207693	1691	V		39.8064	
	8	20.036	28606	289	V		5.4826	
	9	25.41	87883	386	V		16.8437	
	10	26.675	34699	324	V		6.6504	
TOTAL			521757	8863			100	

Fig.10

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
19 October 2000 (19.10.2000)

PCT

(10) International Publication Number
WO 00/61161 A3

(51) International Patent Classification⁷: **A61K 35/78**,
A61P 31/12

(21) International Application Number: PCT/IN00/00046

(22) International Filing Date: 10 April 2000 (10.04.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
405/MAS/99 12 April 1999 (12.04.1999) IN

(71) Applicant (*for all designated States except US*): **UNIVERSITY OF MADRAS** [IN/IN]; Kamarajar Salai, Chepauk, Chennai 600 005 (IN).

(72) Inventor; and

(75) Inventor/Applicant (*for US only*): **THYAGARAJAN, Sadras, Panchatcharam** [IN/IN]; Old No.3, New No. 5, First Street, Nehru Nagar, Adyar, Chennai 600 020 (IN).

(81) Designated States (*national*): AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:

— With international search report.

(88) Date of publication of the international search report:
8 February 2001

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: A PHARMACEUTICAL FORMULATION USEFUL FOR THE TREATMENT OF HEPATITIS B, HEPATITIS C AND OTHER VIRAL INFECTIONS OF THE LIVER AND A PROCESS FOR ITS PREPARATION

(57) Abstract: The invention disclosed in this application relates to a pharmaceutical formulation prepared from the biotyped variety of the medicinal plant, *Phyllanthus amarus* which is useful for the treatment of Hepatitis B (both acute and chronic), Hepatitis C (both acute and chronic) and other related viral infections of the liver with antihepatotoxic and liver cell regenerating potentials and immunomodulating properties. The invention confirms, that when parts of the biotyped variety of the medicinal plant, *Phyllanthus amarus* are extraceted separately with a polar solvent alone, polar solvent and water in specific ratios and water alone and when such extracts are mixed together, the resultant formulation has all the essential antiviral and biological properties, while the individual polar or aqueous extracts alone does not possess one or more of these properties. The present invention also relates to a process for the preparation of the above said new pharmaceutical formulation with biological and chemical standardisation protocols.



WO 00/61161 A3

INTERNATIONAL SEARCH REPORT

Internat. Application No.

PCT/IN 00/00046

A. CLASSIFICATION OF SUBJECT MATTER
 IPC 7 A61K35/78 A61P31/12

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

BIOSIS, EPO-Internal, WPI Data, PAJ, FSTA, MEDLINE, PASCAL, LIFESCIENCES, CAB Data, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>DATABASE BIOSIS 'Online! BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; 1993 YEH SHEAU-FARN ET AL: "Effect of an extract from Phyllanthus amarus on hepatitis B surface antigen gene expression in human hepatoma cells." Database accession no. PREV199395105864 XP002151737 abstract & ANTIVIRAL RESEARCH, vol. 20, no. 3, 1993, pages 185-192, ISSN: 0166-3542</p> <p style="text-align: center;">--- -/--</p>	1-30

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- * & * document member of the same patent family

Date of the actual completion of the international search

1 November 2000

Date of mailing of the international search report

15/11/2000

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
 NL - 2280 HV Rijswijk
 Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
 Fax: (+31-70) 340-3016

Authorized officer

Rempp, G

INTERNATIONAL SEARCH REPORT

Intern: al Application No

PCT/IN 00/00046

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>DATABASE BIOSIS 'Online! BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; 1995 MEIXIA WANG ET AL: "Herbs of the genus Phyllanthus in the treatment of chronic hepatitis B: Observations with three preparations from different geographic sites." Database accession no. PREV199698559653 XP002151738 abstract & JOURNAL OF LABORATORY AND CLINICAL MEDICINE, vol. 126, no. 4, 1995, pages 350-352, ISSN: 0022-2143</p> <p>---</p>	1-30
X	<p>MEHROTRA R ET AL: "IN VITRO EFFECT OF PHYLLANTHUS AMARUS ON HEPATITIS B VIRUS" INDIAN JOURNAL OF MEDICAL RESEARCH, INDIAN COUNCIL OF MEDICAL RESEARCH, NEW DEHLI, IN, vol. 93, March 1991 (1991-03), pages 71-73, XP000929883 ISSN: 0019-5340 the whole document</p> <p>---</p>	1-30
E	<p>WO 00 56347 A (JANARDHAN PALA BUSHANAM ;KUMARAN ALAGANANDAM (IN); PREMILA MANAKKA) 28 September 2000 (2000-09-28) page 1, line 25 -page 4, line 16</p> <p>---</p>	1-30
A	<p>EP 0 890 360 A (DABUR RESEARCH FOUNDATION) 13 January 1999 (1999-01-13)</p> <p>-----</p>	

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.1

Although claims 26-30 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

Continuation of Box I.1

Claims Nos.: 26-30

Rule 39.1(iv) PCT - Method for treatment of the human or animal body by therapy

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/IN 00/00046

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 0056347 A	28-09-2000	NONE	
EP 0890360 A	13-01-1999	NONE	

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ **BLACK BORDERS**
- ☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- ☐ **FADED TEXT OR DRAWING**
- ☐ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- ☐ **SKEWED/SLANTED IMAGES**
- ☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- ☐ **GRAY SCALE DOCUMENTS**
- ☒ **LINES OR MARKS ON ORIGINAL DOCUMENT**
- ☐ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- ☐ **OTHER:** _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.